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THE PEX5-MEDIATED IMPORT PATHWAY: CHARACTERIZATION OF THE TRANSLOCATION STEP

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PRECEITOS LEGAIS

A autora desta tese declara que interveio na concepção e execução do trabalho experimental, na interpretação e redacção dos resultados que, além de incluídos nesta tese, culminaram numa publicação internacional (artigo abaixo indicado), sob o nome de "**Francisco T.**". Além deste artigo, a autora declara ter também participado na elaboração de um artigo de revisão, no qual partilha a primeira autoria.

The author of this thesis declares to have participated in the planning and execution of the experimental work, in the interpretation and preparation of the data which, besides being included in this thesis, were published in an international journal, under the name "**Francisco T.**". Moreover, the author declares to have also participated in the elaboration of a review paper, sharing the first authorship.

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ABSTRACT/RESUMO

ABSTRACT

According to current models, matrix proteins are synthesized in cytosolic ribosomes and bound by the shuttling receptor PEX5. The vast majority of those proteins possess a peroxisomal targeting signal type 1 (PTS1), a tripeptide sequence at their C termini, usually with the sequence S-K-L. A minor part of the peroxisomal matrix proteins contains instead a PTS2 signal, an N-terminal degenerated nonapeptide with the consensus sequence R-(L/V/I/Q)-X_2-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A). The interaction between PEX5 and matrix proteins containing a PTS1 is direct, while the PEX5-PTS2 interaction requires an adaptor protein, PEX7. The cytosolic PEX5-cargo protein complex then interacts with the peroxisomal docking/translocation module (DTM), which ultimately results in PEX5 insertion into this protein machinery. PEX5 is then monoubiquitinated at a conserved cysteine residue, a mandatory modification for its ATP-dependent dislocation back into the cytosol by the Receptor Export Module (REM). Finally, monoubiquitinated PEX5 is deubiquitinated by a conjugation of enzymatic and non-enzymatic mechanisms.

Although our knowledge on the general properties of the PEX5-mediated import pathway is considerable, the exact step of this pathway where cargo proteins are translocated across the organelle membrane is still ill-defined. In an attempt to fill in this gap, the import mechanism of pre-thiolase, a PTS2 protein, has been recently characterized. The data suggest that translocation of pre-thiolase across the peroxisomal membrane occurs before ubiquitination of the DTM-embedded PEX5, presumably during insertion of the receptor into the DTM. However, whether this is a unique feature of PTS2 proteins emerging from the participation of PEX7 in this process, or a general property of the peroxisomal protein import machinery, remained unknown.

The aim of this work was to characterize the translocation mechanism of the major class of matrix proteins, the PTS1 proteins. For this purpose, it was necessary to develop a PTS1-centered *in vitro* import system. A major limitation of such system is the low import yields obtained when using these matrix proteins as reporters. We found that such limitation could be overcome by pre-incubating the reporter protein with recombinant PEX5, prior to the *in vitro* import reaction. Indeed, this resulted in a

remarkable improvement of the import yields of several PTS1 proteins. One of these proteins was Sterol Carrier Protein x (SCPx), a protein involved in the last step of the peroxisomal β-oxidation of fatty acids. Using this strategy, we have characterized the import mechanism of SCPx. Our results show that the *in vitro* import efficiencies of SCPx were not affected by the use of AMP-PNP or by apyrase treatment, although PEX5 export and PEX5 monoubiquitination, respectively, are blocked under these conditions. Moreover, a PEX5 mutated version which is not a substrate for monoubiquitination, and consequently is not a substrate for the REM, was as efficient as the normal PEX5 in transporting SCPx to the peroxisome. Altogether, these data demonstrate that neither PEX5 monoubiquitination nor its export are required for SCPx import. Similar results were obtained for 2,4-dienoyl-CoA reductase (DECR2), another PTS1 protein, thus suggesting that this is a general property of the mechanism of PTS1 protein translocation. Peroxisome fractionation analyses indicated that at least a fraction of the imported SCPx was completely translocated to the peroxisomal matrix, in a process, again, requiring neither cytosolic ATP hydrolysis nor ubiquitination of PEX5. The PTS1-centered *in vitro* import system developed in this work also allowed us to observe for the first time a mechanistically distinct docking step of the PEX5-cargo complex at the DTM. These results support a model in which translocation of proteins across the peroxisomal membrane occurs before the first cytosolic ATP-dependent step (*i.e.*, before PEX5 ubiquitination), concomitantly with the insertion of the receptor into the docking/translocation machinery.

RESUMO

Segundo os modelos actuais, as proteínas da matriz peroxissomal são sintetizadas em ribossomas citosólicos e endereçadas para o organelo pelo receptor peroxisomal PEX5. A maioria das proteínas matriciais contêm um sinal de endereçamento peroxisomal do tipo 1 (PTS1), um tripéptido presente no C-terminal, normalmente com a sequência S-K-L. Uma minoria possui um sinal PTS2, um nonapéptido degenerado presente no N-terminal destas proteínas, cuja sequência consenso é R-(L/V/I/Q)-X_2-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A). A PEX5 interage directamente com proteínas matriciais que contenham um PTS1, enquanto a interacção com proteínas PTS2 requer uma proteína adaptadora, a PEX7. Na membrana peroxissomal, o complexo PEX5-proteína matricial interage com o módulo peroxissomal de *docking* e translocação de proteínas (DTM), conduzindo à inserção da PEX5 nesta maquinaria proteica. A PEX5 é posteriormente monoubiquitinada numa cisteína conservada, uma modificação obrigatória para a sua remoção do DTM para o citosol pelo Módulo de Exportação do Receptor (REM), num processo dependente de ATP. Finalmente, a PEX5 monoubiquitinada, é desubiquitinada no citosol por uma combinação de mecanismos enzimáticos e não enzimáticos.

Apesar do nosso conhecimento sobre a via de importação mediada pela PEX5 ser já bastante detalhado, a informação relativa ao passo exacto desta via onde as proteínas da matriz peroxisomal são translocadas através da membrana do organelo ainda é escassa. Recentemente, numa tentativa de esclarecer este ponto, foi caracterizado o mecanismo de importação da pré-tiolase, uma proteína com um sinal PTS2. Os dados obtidos sugerem que a translocação da pré-tiolase através da membrana peroxisomal ocorre antes do passo de ubiquitinação da PEX5, presumivelmente durante a sua inserção no DTM. No entanto, se isto é uma característica única da via de importação de proteínas com um sinal PTS2 que emerge do envolvimento da PEX7 ou uma propriedade geral do sistema de importação peroxissomal, permanecia por descobrir.

O objectivo deste trabalho foi caracterizar o mecanismo de translocação da classe principal de proteínas matriciais, as proteínas que contêm um PTS1. Para o efeito, foi necessário desenvolver um sistema de importação *in vitro* centrado numa

proteína PTS1. A grande limitação deste sistema é o baixo rendimentos de importação quando se usam proteínas matriciais PTS1 como proteínas repórter. Descobrimos que essa limitação poderia ser ultrapassada pré-incubando a proteína repórter com PEX5 recombinante antes da reacção de importação *in vitro*. De facto, isto resultou num aumento significativo dos rendimentos de importação de várias proteínas PTS1. Uma destas proteínas é a *Sterol Carrier Protein x* (SCPx), uma proteína envolvida no último passo da via de β-oxidação de ácidos gordos. Usando esta estratégia, caracterizámos o mecanismo de importação da SCPx. Os resultados obtidos demonstram que as eficiências da sua importação não foram afectadas pelo uso de AMP-PNP, nem pelo tratamento com apirase, apesar da exportação e monoubiquitinação da PEX5, respectivamente, estarem bloqueadas nestas condições. Adicionalmente, uma versão mutada da PEX5 incapaz de ser monoubiquitinada e que, consequentemente, não é exportada pelo REM, foi tão eficiente como a PEX5 normal no transporte da SCPx para o peroxissoma. Em conjunto, os dados obtidos demonstram que nem a monoubiquitinação da PEX5, nem a sua exportação são necessárias para a importação da SCPx. Resultados semelhantes foram obtidos para a 2,4-dienoil-CoA redutase (DECR2), outra proteína PTS1, sugerindo que esta é uma propriedade geral do processo de translocação de proteínas PTS1. Análises de fraccionamento peroxissomal demonstraram que pelo menos uma fracção da SCPx importada já tinha sido completamente translocada para a matriz peroxissomal, num processo, novamente, independente da hidrólise de ATP citosólico e da monoubiquitinação da PEX5. O sistema de importação *in vitro* centrado numa proteína PTS1 desenvolvido neste trabalho, permitiu ainda, pela primeira vez, a observação de um passo mecanisticamente distinto de *docking* do complexo PEX5-proteína matricial no DTM. Estes resultados suportam um modelo no qual a translocação de proteínas através da membrana peroxissomal ocorre antes do primeiro passo dependente de ATP citosólico (*i.e.*, antes da ubiquitinação da PEX5), concomitantemente com a inserção do receptor na maquinaria peroxisomal de *docking* e translocação.

ABBREVIATIONS

AAA	ATPases associated with diverse cellular activities
ABC	ATP-Binding Cassette
ACOX1	Acyl-CoA oxidase 1
ADHAPS	Alkyl-DHAP synthase
AGT	Alanine glyoxylate aminotransferase
ALDP	Adrenoleukodystrophy protein
AMACR	2-methylacyl-CoA racemase
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CHO	Chinese hamster ovary
DBP	D-bifunctional protein
DECR2	2,4-dienoyl-CoA reductase
DHAP	Dihydroxyacetone phosphate
DHAPAT	DHAP acyltransferase
DTM	Docking/translocation module
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E-64	N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FIS1	Fission 1
GSH	Glutathione
GST	Gluthathione-S-transferase
H. polymorpha	*Hansenula polymorpha*
IAA	Iodoacetamide
IgG	Immunoglobulin G
MFF	Mitochondrial fission factor

MOPS	4-morpholinepropanesulfonic acid
mPTS	Membrane peroxisomal targeting signal
NALD	Neonatal Adrenoleukodystrophy
NTP	Nucleoside triphosphate
PAGE	Polyacrylamide gel electrophoresis
PBDs	Peroxisome biogenesis disorders
PEX	Peroxin
PH1	Primary Hyperoxaluria type 1
PHYH	Phytanoyl-CoA hydroxylase
PIM	Peroxisomal import machinery
PK	Proteinase K
PMP	Peroxisome membrane protein
PMSF	Phenylmethylsulfonyl fluoride
PNS	Postnuclear supernatant
PTS1	Peroxisomal targeting signal 1
PTS2	Peroxisomal targeting signal 2
REM	Receptor export module
RING	Really interesting new gene
S. cerevisiae	*Saccharomyces cerevisiae*
SCP2	Sterol carrier protein 2
SCPx	Sterol carrier protein x
SDS	Sodium dodecylsulfate
SEM	Buffer containing sucrose, EDTA and MOPS
SH3	Src homology 3 domain
TCA	Trichloroacetic acid
TPR	Tetratricopeptide repeats
Tris	Tris(hydroxymethyl)aminomethane
Ub	Ubiquitin
Ubal	Ubiquitin aldehyde
Ub-PEX5	Monoubiquitinated PEX5
VLCFA	Very long chain fatty acid
X-ALD	X-linked Adrenoleukodystrophy

WD	Tryptophan-aspartate repeat
Y. lipolytica	*Yarrowia lipolytica*
ZS	Zellweger syndrome
ZSDs	Zellweger spectrum disorders

1. INTRODUCTION

1.1. Peroxisome structure and function

Peroxisomes are unique organelles present in almost all eukaryotic cells. They were first described in 1954, by Rhodin, as microbodies (Rhodin, 1954). Peroxisomes owe their current name to De Duve and Baudhuin who, in 1966, defined them as a cellular compartment harboring at least one oxidase, forming hydrogen peroxide, and catalase, the enzyme that decomposes it (De Duve & Baudhuin, 1966). Peroxisomes can display a very heterogeneous morphology that varies among different species, tissues and prevailing environmental or development conditions (reviewed in (Lazarow & Fujiki, 1985; Purdue & Lazarow, 2001; Schrader & Fahimi, 2006; Fransen, 2012)). Typically, they are spherical or ovoid, exhibiting a size range between 0.1-1 μm in diameter. However, they may also appear as elongated, tubular, or reticular structures (Schrader *et al*, 1996). These single-membrane organelles lack DNA or an independent protein synthesis machinery (Kamiryo *et al*, 1982; Douglass *et al*, 1973; Leighton *et al*, 1968). In electron microscopy, they are structurally characterized by a very electron-dense protein-rich granular matrix. In liver and renal cells from several organisms they may contain crystalline inclusions comprising urate oxidase or other enzymes (Figure 1) (Leighton *et al*, 1968; Tsukada *et al*, 1966; Zaar & Fahimi, 1991).

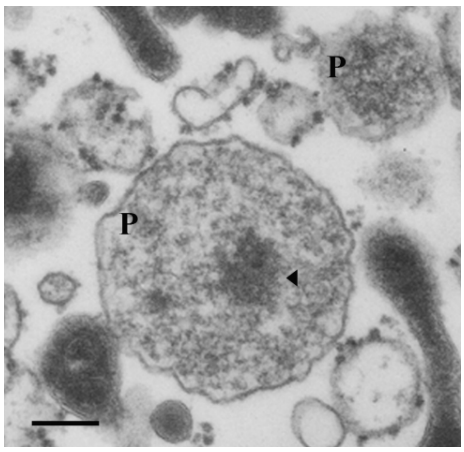


Figure 1. Rat liver peroxisomes.
Electron micrograph of peroxisomes (P) from a rat liver postnuclear supernatant where crystalline cores of urate oxidase (▶) can be observed. *Scale bar*, 0.2 μm (micrograph kindly taken by Prof. Dr. Manuel Teixeira da Silva, IBMC, Porto, Portugal).

Peroxisomes are very often referred to as “multipurpose organelles” due to their involvement in several metabolic and non-metabolic pathways (see Table 1). They are highly versatile and dynamic, and although often specialized according to the organism and cell type, all peroxisomes share two common conserved features: the metabolism of hydrogen peroxide and β-oxidation of fatty acids (Cooper & Beevers, 1969; Lazarow & De Duve, 1976; Breidenbach *et al*, 1968; Kawamoto *et al*, 1978). In mammalian cells, β-oxidation is distributed between peroxisomes and mitochondria. Short, medium and some long chain fatty acids are exclusively oxidized in mitochondria, whereas very long chain fatty acids (VLCFA) can only be processed in peroxisomes (Wanders & Waterham, 2006a; Poirier *et al*, 2006). In fungi and plants, β-oxidation is instead a strictly peroxisomal process (Poirier *et al*, 2006). Fatty acid α-oxidation is another peroxisomal process in mammals which is required for the degradation of branched-chain fatty acids, like phytanic acid (Wanders *et al*, 2011; Croes *et al*, 1996; Jansen *et al*, 1996; Mihalik *et al*, 1995). In agreement with their diverse metabolic function, peroxisomes display a striking heterogeneity regarding their enzyme content (Islinger *et al*, 2010; Hu *et al*, 2012; Gabaldón, 2010). Actually, some highly specialized peroxisomes have even different designations. For instance, peroxisomes of germinating seeds are often designated glyoxysomes due to their enrichment in enzymes of the glyoxylate cycle (Olsen, 1998). Similarly, peroxisomes of trypanosomes are known as glycosomes, as they contain several enzymes involved in glycolysis (Michels *et al*, 2006). Another example is provided by the Woronin bodies which are very specialized peroxisomes, whose function is to seal septal pores, preventing cytoplasmic bleeding when hyphae of filamentous fungi are damaged (Pieuchot & Jedd, 2012). Among other peroxisomal functions are some species/tissue-specific tasks (see also Table 1). In plants, for instance, peroxisomes also participate in the synthesis of jasmonic acid, auxin and biotin, and are involved both in photorespiration in leaves and nitrogen transport in roots (Olsen, 1998; Hu *et al*, 2012; Islinger *et al*, 2012; Tanabe *et al*, 2011). In mammals, peroxisomes are also involved in the synthesis of bile acids and plasmalogens (Wanders, 2013; Fidaleo, 2010; Fransen, 2012; Terlecky *et al*, 2012; Islinger *et al*, 2012). In some fungi, peroxisomes also participate in the synthesis of β-lactam antibiotics (*Penicillium*

chrysogenum) and biotin (*Aspergillus oryzae*) (Tanabe *et al*, 2011; Islinger *et al*, 2012). Finally, in methylotrophic yeasts, peroxisomes are crucial for the growth in methanol as a sole source of carbon and energy (Yurimoto *et al*, 2011; Islinger *et al*, 2012). In many of the biochemical pathways peroxisomes do not work alone. Rather they act as members of a coordinated cellular machinery, physically and functionally interacting with other subcellular compartments, namely mitochondria and the endoplasmic reticulum (ER) (Bonekamp & Schrader, 2012; Braverman & Moser, 2012; Camões *et al*, 2009; Van Veldhoven, 2010; Kohlwein *et al*, 2013; Wanders, 2013).

Recently, a non-metabolic biological task has been added to the list of mammalian peroxisomes functions. Specifically, it was suggested that peroxisomes can act as signaling platforms, involved not only in transient and complex regulatory interactions of cellular processes but also in antiviral signaling, providing a rapid and short-term protection during viral infection (Dixit *et al*, 2010; Ribeiro *et al*, 2012; Zhang *et al*, 2013).

Table 1. Diversity of peroxisomal functions

Peroxisomal Functions
Fatty acid β-oxidation
Degradation of polyamines
Detoxification of Reactive Oxygen Species (ROS)
Glyoxylate cycle
Biosynthesis of etherphospholipids
Fatty acid α-oxidation
Synthesis of bile acids
Synthesis of pheromones
Viral innate immune defense
Glycolysis
Degradation of icosanoids
Degradation of xenobiotics
Photorespiration
Nitrogen transport
Synthesis of jasmonic acid and auxin
Synthesis of toxins
Synthesis of β-lactam antibiotics
Methanol and methylamines metabolism
Synthesis of docosahexaenoic acids
Synthesis of biotin

Adapted from (Fransen, 2012; Hu *et al*, 2012; Terlecky *et al*, 2012; Fidaleo, 2010; Islinger *et al*, 2012; Olsen, 1998; Bartoszewska *et al*, 2011; Kunze & Hartig, 2013; Kunze *et al*, 2006; Wanders, 2013).

1.2. Peroxisomes and disease

Although peroxisomes were discovered more than fifty years ago, they became a pivotal subject in the scientific community only after the identification of the so-called, peroxisomal disorders (see Table 2). The first link between peroxisomes and an inherited lethal disorder was established in 1973, with the discovery that patients affected with the Zellweger cerebro-hepato-renal syndrome lack peroxisomes in hepatocytes and renal tubule cells (Goldfischer *et al*, 1973). Many other genetic disorders ethiologically related to peroxisome function were discovered in the 1980s and 1990s, thus establishing the real importance of peroxisomes in human health and disease (Goldfischer *et al*, 1973; Wanders *et al*, 1988; Zellweger *et al*, 1988; Heikoop *et al*, 1990; Biardi *et al*, 1994; Fujiki, 2000; Wanders, 2004b; Delille *et al*, 2006; Waterham & Ebberink, 2012).

Table 2. Peroxisomal disorders and metabolic pathways affected

Peroxisomal Disorders	Metabolic Pathway Affected
Peroxisomal single Enzyme Deficiencies (PEDs)	
2-Methylacyl-CoA racemase (AMACR) deficiency (AMACR deficiency)	β-oxidation of fatty acids
Acyl-CoA oxidase (ACOX1) deficiency (ACOX1 deficiency)	
D-Bifunctional protein (DBP) deficiency (DBP deficiency)	
Sterol Carrier Protein x (SCPx) deficiency (SCPx deficiency)	
X-linked Adrenoleukodystrophy (ALDP deficiency)	
Refsum disease (PHYH deficiency)	α-oxidation of fatty acids
Rhizomelic chondrodysplasia punctata Type 2 (DHAPAT deficiency)	Ether phospholipid synthesis
Rhizomelic chondrodysplasia punctata Type 3 (ADHAPS deficiency)	
Acatalasaemia (catalase deficiency)	H_2O_2 metabolism
Primary Hyperoxaluria Type 1 (AGT deficiency)	Glyoxylate detoxification
Bile acid-CoA:amino acid N-acyltransferase deficiency (BAAT deficiency)	Bile acid synthesis
Peroxisome Biogenesis Disorders (PBDs)	
Zellweger Spectrum Disorders (ZSDs)	Several pathways affected
Zellweger Syndrome (ZS)	
Neonatal Adrenoleukodystrophy (NALD)	
Infantile Refsum Disease (IRD)	
PEX11β deficiency	
Rhizomelic chondrodysplasia punctata (RCDP) Type 1	

Adapted from (Delille *et al*, 2006; Fidaleo, 2010; Wanders, 2013; Thoms & Gärtner, 2012). Abbreviations: ALDP, adrenoleukodystrophy protein; PHYH, phytanoyl-CoA hydroxylase; DHAPAT, dihydroxyacetone phosphate (DHAP) acyltransferase; ADHAPS, alkyl- DHAP synthase; AGT, Alanine glyoxylate aminotransferase.

Peroxisomal disorders represent a spectrum of genetically inherited metabolic diseases that are characterized by the lack of peroxisomes or an impairment of one or more peroxisomal functions (Wanders, 2004b). They are rare multisystemic disorders frequently affecting the nervous system (Barry & O'Keeffe, 2013; Poll-The & Gartner, 2012). The severity of these diseases varies according to the extent of the impairment of peroxisome functions.

Peroxisomal disorders can be classified into two major groups: 1) the peroxisomal single enzyme deficiencies, and 2) the peroxisome biogenesis disorders (reviewed in (Delille *et al*, 2006; Gould & Valle, 2000; Sacksteder & Gould, 2000; Steinberg *et al*, 2006; Wanders, 2004a; Fidaleo, 2010)).

1.2.1. Peroxisomal single enzyme deficiencies

Peroxisomal single enzyme deficiencies (see Table 2) result from mutation in a gene encoding a peroxisomal enzyme or transporter thus affecting one specific peroxisomal function, such as α- or β-oxidation of fatty acids, ether phospholipid synthesis, glyoxylate detoxification or H_2O_2 metabolism (Wanders & Waterham, 2006b). In these disorders, the peroxisomes are intact and functional, except for the single metabolic pathway affected by the mutation.

X-linked Adrenoleukodystrophy (X-ALD) is the most common peroxisomal single enzyme deficiency, with an estimated birth incidence of 1 in 17,000 newborns (Engelen *et al*, 2012). X-ALD is a neurodegenerative and progressive disorder caused by mutations in the ABCD1 gene (reviewed in (Wang *et al*, 2011) and (Kemp & Wanders, 2010)). The ABCD1 gene encodes a peroxisomal ATP-Binding Cassette (ABC) transporter protein, the Adrenoleukodystrophy protein (ALDP), whose function is to transport CoA thioesters of VLCFA from the cytosol to the peroxisomal matrix (Mosser *et al*, 1993; Wiesinger *et al*, 2013). ALDP deficiency thus impairs VLCFA β-oxidation and leads to an accumulation of VLCFA in cells and tissues (Moser *et al*, 1999). By largely undefined mechanisms, this accumulation results in neurological degeneration by nerve demyelination (Asheuer *et al*, 2005). The nervous system loses the ability to fully coordinate nerve impulses and, consequently, X-ALD patients can experience motor skill loss, sensory deficit, seizures, dementia and blindness

(Kemp *et al*, 2012). X-ALD treatments are very limited and essentially symptomatic. Some dietary treatments, such as Lorenzo's oil combined with a VLCFA-poor diet, reduce VLCFA levels but do not stop the disease's progression (Ferrer *et al*, 2010; Aubourg *et al*, 1993). Presently, hematopoietic stem cell transplantation is the only curative approach, but it is only effective in patients with early stages of cerebral symptoms (Peters *et al*, 2004; Ferrer *et al*, 2010).

Another example of a peroxisomal single enzyme deficiency is provided by Primary Hyperoxaluria type 1 (PH1), a disease caused by a deficiency in peroxisomal alanine glyoxylate aminotransferase (AGT), an enzyme that catalyzes the conversion of glyoxylate into glycine (Danpure & Jennings, 1986). In the absence of AGT, peroxisomal glyoxylate accumulates and is reduced to glycolate or oxidized to oxalate. Unlike glycolate, which is soluble and therefore can be excreted in urine, oxalate precipitates as calcium oxalate and accumulates in liver and other organs, especially in the kidney (Wanders & Waterham, 2006b; Danpure, 2006). In some cases, PH1 is not caused by AGT loss of catalytic activity but instead by its mislocalization to mitochondria (Danpure *et al*, 2003). PH1 is characterized by hyperoxaluria associated or not with hyperglycolic aciduria, calcium oxalate urolithiasis or nephrocalcinosis and, as mentioned above, progressive loss of renal function over time. In contrast to other peroxisomal disorders, there are some therapeutic options for the treatment of PH1 which target the reduction of oxalate synthesis and the increase of calcium oxalate solubility (reviewed in (Salido *et al*, 2012)).

1.2.2. Peroxisome biogenesis disorders (PBDs)

The second group of peroxisomal disorders are the PBDs (Table 2). These diseases occur in approximately 1:50,000 live births in the United States (Steinberg *et al*, 2006; Barry & O'Keeffe, 2013) and represent a spectrum of autosomal recessive metabolic disorders that are caused by mutations in genes encoding peroxins (PEX), *i.e.*, peroxisomal proteins required for peroxisome biogenesis and maintenance (see Table 3) (Fujiki *et al*, 2012b; Waterham & Ebberink, 2012). Therefore, when a *PEX* gene is affected, several or all peroxisomal functions are impaired and peroxisomes

may be completely absent. In many cases, it is still possible to observe peroxisomal remnants in patient cells. These structures are known as peroxisomal ghosts (Brown & Baker, 2003; Purdue & Lazarow, 2001; Santos *et al*, 1988b). Peroxisomal ghosts are empty organelles that lack all, or almost all, their matrix content due to a defect in peroxisome matrix protein import (even though they possess the normal repertoire of membrane proteins). The effects of the loss of functional peroxisomes include an accumulation of toxic peroxisomal substrates, such as VLCFA or phytanic acid, and a depletion of essential metabolites normally produced in peroxisomes (Lee & Raymond, 2013; Sacksteder & Gould, 2000; Steinberg *et al*, 2006). One example of the latter are the plasmalogens whose complete or major depletion is at the basis of both bone and brain deficiencies observed in PBDs patients (Braverman & Moser, 2012).

PBDs can be caused by defects in any of, at least, fourteen different *PEX* genes (see Table 3) (Fidaleo, 2010; Fujiki *et al*, 2012b; Gould & Valle, 2000; Steinberg *et al*, 2006). Thirteen of these genes have been implicated in the so-called Zellweger Syndrome Spectrum disorders (ZSDs), while mutations in the remaining one (the *PEX7* gene) are the cause of the disorder Rhizomelic Chondrodysplasia Punctata (RCDP) type 1 (Waterham & Ebberink, 2012). The ZSDs include three overlapping clinical entities that share liver disease, neurodevelopmental delay, retinopathy and perceptive deafness but differ in the severity of symptoms (Brosius & Gartner, 2002; Wanders & Waterham, 2005; Waterham & Ebberink, 2012). The Zellweger Syndrome (ZS), or cerebro-hepato-renal syndrome, is the most severe form of the spectrum. It is characterized by the absence of functional peroxisomes in all cells. ZS patients present hypotonia, craniofacial dysmorphism, weakness, severe brain dysfunction associated with neuronal migration defects at birth and usually die within the first year of age (Braverman *et al*, 2013; Brosius & Gartner, 2002). Neonatal adrenoleukodystrophy (NALD) is an intermediate form of ZSDs, with patients presenting clinical symptoms similar, yet less severe, to those of ZS patients. The life expectancy is also longer in NALD than in ZS (Waterham & Ebberink, 2012; Wanders & Waterham, 2005; Braverman *et al*, 2013). The mildest form of the ZSDs is the Infantile Refsum disease (IRD). Patients with IRD do not show neuronal migration

disorder or progressive white matter disease (Braverman *et al*, 2013; Waterham & Ebberink, 2012; Poll-The & Gartner, 2012). Clinical presentations of IRD include hypotonia, hearing loss, vision impairment, and developmental delay (Braverman *et al*, 2013; Wanders & Waterham, 2005; Waterham & Ebberink, 2012; Poll-The & Gartner, 2012). IRD is a slow progression disorder, with patients frequently surviving until 30 years of age. Similarly to NALD, IRD patients may present some level of mosaicism with some cells presenting residual peroxisomes (Matsui *et al*, 2012; Steinberg *et al*, 2006; Matsumoto *et al*, 2001).

Recently, a new mild ZSD was identified in a Dutch patient presenting atypical biochemical features for a PBD. Specifically, this patient had normal biochemical peroxisome parameters, including plasma concentrations of VLCFA, phytanic and pristanic acid (Ebberink *et al*, 2012). However, analyses of peroxisome morphology in skin fibroblasts of this patient revealed elongated and enlarged organelles. After analysis of several candidate genes, a homozygous point mutation in the *PEX11β* gene was found (Ebberink *et al*, 2012), in agreement with the known function of PEX11β in peroxisome proliferation and division (Ebberink *et al*, 2012; Koch *et al*, 2010; Huber *et al*, 2012).

The last of PBDs, RCDP type 1, is caused by mutations in the *PEX7* gene (Braverman *et al*, 1997; Purdue *et al*, 1997). These mutations result in the mistargeting of only a subset of peroxisomal proteins, producing a biochemical, cellular, and clinical phenotype distinct from the ZSDs (Braverman *et al*, 1997; Purdue *et al*, 1997; Mohamadynejad *et al*, 2013). Although peroxisomes present a normal morphology, several functions are compromised, namely plasmalogen synthesis and α-oxidation of fatty acids. RCDP type 1 is characterized by bone abnormalities (shortening of the upper extremities – rhizomelia; stippled epiphyses – chondrodysplasia punctate; low stature; facial dysmorphism), cataracts, microcephaly and psychomotor retardation (Braverman *et al*, 2013; Jansen *et al*, 2004).

Table 3. Genes affected in Peroxisome Biogenesis Disorders (PBDs)

Gene	Phenotype	Peroxisome Ghosts	Pathway affected
PEX1	ZS, NALD, IRD	+	Matrix protein import (Receptor recycling)
PEX2	ZS, IRD	+	Matrix protein import (Receptor recycling)
PEX3	ZS	-	Membrane protein import
PEX5	ZS, NALD	+	Matrix protein import
PEX6	ZS, NALD	+	Matrix protein import (Receptor recycling)
PEX7	RCDP type1	+	Matrix protein import
PEX10	ZS, NALD	+	Matrix protein import (Receptor recycling)
PEX11β	mild ZSS	+	Peroxisome proliferation/division
PEX12	ZS, NALD, IRD	+	Matrix protein import (Receptor recycling)
PEX13	ZS, NALD	+	Matrix protein import (Docking/Translocation)
PEX14	ZS	+	Matrix protein import (Docking/Translocation)
PEX16	ZS	-	Membrane protein import
PEX19	ZS	-	Membrane protein import
PEX26	ZS, NALD, IRD	+	Matrix protein import (Receptor recycling)

Adapted from: (Fujiki *et al*, 2012b; Nagotu *et al*, 2012; Waterham & Ebberink, 2012; Thoms & Gärtner, 2012).

1.3. Peroxisome biogenesis

It is generally accepted that peroxisome biogenesis involves, at least conceptually, three steps: 1) biogenesis/assembly of the peroxisomal membrane; 2) import of all of the matrix content; and 3) peroxisome proliferation.

The identification of the proteins (and corresponding genes) involved in these three aspects of peroxisome biogenesis was essentially performed using two main strategies. The vast majority of the *PEX* genes were identified by genetic complementation studies in yeast and Chinese Hamster Ovary (CHO) cell mutants (Tsukamoto *et al*, 1990; Fujiki *et al*, 2000; Erdmann *et al*, 1989; Liu *et al*, 1992; Gould *et al*, 1992). Homology searches by screening the human expressed sequence tag database using yeast PEX genes further contributed for the isolation of human ortholog genes (Weller *et al*, 2003; Fujiki *et al*, 2006). More recently, another strategy has been used to identify other components involved in peroxisome biogenesis: the biochemical isolation of enzyme activities using standard protein purification procedures. This strategy allowed the identification of components of the machinery that also participate in other cellular pathways, namely the mammalian E2s E2D1/2/3,

the mammalian deubiquitinase USP9X, the yeast deubiquitinase UBP15 and mammalian AWP1 ((Grou *et al*, 2008, 2012; Debelyy *et al*, 2011; Miyata *et al*, 2012; Bozza & Zhuang, 2011); see also below).

Presently, more than thirty peroxins are known in different organisms (see Table 4). Strikingly, however, only sixteen of them are present in mammals (Kiel *et al*, 2006; Schlüter *et al*, 2006; Zolman *et al*, 2005; Goto *et al*, 2011). Apparently, evolution led to a simpler architecture of the peroxisomal biogenesis machinery (see Figure 1 in (Grou *et al*, 2009a)). This simplification was probably achieved by the acquisition of extra functions by the corresponding mammalian/plant proteins. PEX18/21 (*Saccharomyces cerevisae*) and PEX20 (other yeasts and fungi) (see also section 1.3.3.), for example, are species-specific peroxins absent in mammals and plants, whose role in the targeting of a subset of matrix proteins has been embraced by the receptor PEX5 of these organisms (Lazarow, 2006; Schliebs & Kunau, 2006). Similarly, yeasts and plants possess a peroxisomal dedicated E2, PEX4 and its membrane anchor PEX22, while in mammals, the E2 peroxisomal function has been embraced by the multipurpose cytosolic E2s, E2D1/2/3 (UbcH5a/b/c in mammals) (Grou *et al*, 2008; Zolman *et al*, 2005; Platta *et al*, 2007; Williams *et al*, 2007; Koller *et al*, 1999; Kiel *et al*, 2006; Schlüter *et al*, 2006). Notwithstanding these differences, it seems that the basic design of the peroxisomal biogenesis machinery was fairly conserved throughout evolution.

All peroxisomal matrix and membrane proteins are encoded by nuclear genes (Lazarow & Fujiki, 1985). After synthesis on cytosolic ribosomes, proteins need to be targeted to the peroxisome. The first hint which suggested that different machineries were involved in the targeting of matrix and membrane proteins came from the observation that in many Zellweger patients (section 1.2.2.) cells possess peroxisomal “ghosts”. These structures contain all membrane proteins but no matrix content (Santos *et al*, 1988b, 1988a). In contrast, in a few Zellweger patients no peroxisomal ghosts could be detected in their cells, suggesting a defect in peroxisomal membrane biogenesis. The observation of these two distinct phenotypes pointed for two separated protein import pathways in peroxisomal biogenesis: one for peroxisomal matrix proteins and another for membrane proteins.

Details on these two protein targeting pathways will be presented in sections 1.3.1. and 1.3.3. Peroxisome proliferation will be briefly described in section 1.3.2.

Table 4. Proteins involved in peroxisome biogenesis: features and functions.

	PEX protein	Localization	Organism	Functions and properties
Matrix protein import	PEX5	Cyt / Memb	M, P, F, Y	PTS1 and PTS2 targeting; IDD, TPRs
	PEX7	Cyt / Memb	M, P, F, Y	PTS2 targeting; adaptor protein; WD repeats
	PEX18	Cyt / Memb	Y	PTS2 targeting
	PEX20	Cyt / Memb	F, Y	PTS2 targeting
	PEX21	Cyt / Memb	Y	PTS2 targeting
	PEX13	Memb	M, P, F, Y	SH3
	PEX14	Memb	M, P, F, Y	Coiled-coil
	PEX17	Memb	Y	Coiled-coil
	PEX33 (PEX14/17)	Memb	F	Coiled-coil
	PEX8	Matrix/ Memb	F, Y	
	PEX2	Memb	M, P, F, Y	E3; RING zing-binding domain
	PEX10	Memb	M, P, F, Y	E3; RING zing-binding domain
	PEX12	Memb	M, P, F, Y	E3; RING zing-binding domain
	PEX1	Cyt / Memb	M, P, F, Y	Receptor export; AAA ATPase
	PEX6	Cyt / Memb	M, P, F, Y	Receptor export; AAA ATPase
	PEX15	Memb	F, Y	PEX1/PEX6 membrane anchor
	PEX26	Memb	M, F, Y	PEX1/PEX6 membrane anchor
	APEM9	Memb	P	PEX1/PEX6 membrane anchor
	AWP1	Cyt	M	Adaptor protein; A20 and AN1 zinc finger domains
	PEX4	Memb/Cyt	P, F, Y	E2
	PEX22	Memb	P, F, Y	PEX4 membrane anchor
	E2D1/2/3	Cyt	M	E2
	UBP15	Memb/Cyt	Sc	DUB
	USP9X	Cyt	M	DUB
Membrane protein import	PEX3	Memb	M, P, F, Y	
	PEX16	Memb	M, P, F, Y	
	PEX19	Cyt / Memb	M, P, F, Y	PMP targeting; Farnesylation motif
Proliferation and inheritance	PEX11	Memb	M, P, F, Y	
	PEX23	Memb	F, Y	Dysferlin
	PEX24	Memb	F, Y	
	PEX25	Memb	Y	
	PEX27	Memb	Sc	
	PEX28	Memb	Sc	
	PEX29	Memb	Y	
	PEX30	Memb	Sc	Dysferlin
	PEX31	Memb	Sc	Dysferlin
	PEX32	Memb	Y	Dysferlin
	PEX34	Memb	Sc	

Abbreviations: Cyt, cytosol; Memb, membrane; M, mammals; P, plants; F, fungi; Y, yeast; Sc, *S. cerevisiae*; PTS1, peroxisomal targeting signal 1; PTS2, peroxisomal targeting signal 2; IDD, intrinsic disordered domain; TPRs, tetratricopeptide repeats; WD, Tryptophan-aspartate repeat; SH3, Src homology 3; RING, really interesting new gene; E3, ubiquitin ligase; Ub, ubiquitin; AAA, ATPases associated with diverse cellular activities; E2, ubiquitin-conjugation enzyme; DUB, desubiquitinase; PMP, peroxisomal membrane protein.

1.3.1. Peroxisomal membrane biogenesis

The import of peroxisomal membrane proteins (PMPs) is, in parallel with the origin of the peroxisomal membrane, a very controversial issue. Depending on the

different views concerning the formation of the organelle, several models of PMP translation and sorting have been proposed (Beevers, 1979; Fujiki *et al*, 1984; Rachubinski *et al*, 1984; Lazarow & Fujiki, 1985; Purdue & Lazarow, 2001; Dimitrov *et al*, 2013). It is generally accepted that peroxisomes can be originated both by growth and division, as autonomous organelles, as well as, at least under certain circumstances, by *de novo* formation via the ER. However, the physiological relevance of the *de novo* formation and the contribution of each of the pathways for the peroxisome biogenesis is still controversial and unknown (Motley & Hettema, 2007; Nagotu *et al*, 2008; Kim *et al*, 2006; van der Zand *et al*, 2012).

So far, three proteins have been identified as the key components of the membrane biogenesis machinery, namely PEX3, PEX19 and, in mammals, also PEX16 (Fujiki *et al*, 2006; Honsho *et al*, 2002). Deletion of the genes encoding these proteins results in total loss of peroxisomal structures, suggesting that they play a key role in the organelle formation (South & Gould, 1999; Matsuzono *et al*, 1999; Hettema *et al*, 2000; Shimozawa *et al*, 2000; Muntau *et al*, 2000).

Since PEX19 is present in all peroxisome-containing organisms, displays a dual peroxisomal/cytosolic localization and has the ability to interact with most PMPs, it has been suggested that this protein is a shuttling receptor for PMPs (Sacksteder *et al*, 2000; Götte *et al*, 1998; Snyder *et al*, 1999; Jones *et al*, 2004; Fransen *et al*, 2001; Shibata *et al*, 2004; Fransen *et al*, 2005; Halbach *et al*, 2006; Matsuzono *et al*, 2006). According to this idea, PEX19 recognizes newly synthesized PMPs in the cytosol and transports them to the peroxisome membrane. PEX19 interacts directly with PMPs via their membrane peroxisomal targeting signal (mPTS) (Sacksteder *et al*, 2000; Halbach *et al*, 2006; Van Ael & Fransen, 2006; Yernaux *et al*, 2006; Rottensteiner *et al*, 2004; Saveria *et al*, 2007). These are small weakly conserved protein domains, frequently comprising a cluster of basic residues predicted to form an α-helix, adjacent to one or more transmembrane segments (Dyer *et al*, 1996; Baerends *et al*, 2000; Honsho & Fujiki, 2001; Jones *et al*, 2001; Wang *et al*, 2001; Rottensteiner *et al*, 2004). PEX19 was suggested to also act as a chaperone, preventing aggregation and degradation of the newly synthesized PMPs, probably by shielding their hydrophobic protein surfaces (Jones *et al*, 2004). Accordingly, it was demonstrated that PEX19

binds PMPs during or immediately after translation, and escorts them to the peroxisomal membrane, where it interacts with the docking protein, PEX3 (Götte *et al*, 1998; Fang *et al*, 2004; Hoepfner *et al*, 2005; Muntau *et al*, 2003; Pinto *et al*, 2006). In agreement, cargo-loaded PEX19 displays a much higher affinity for PEX3 than PEX19 alone (Pinto *et al*, 2006). The interaction of the PEX19-PMP protein complex with PEX3 ultimately results in the insertion of the PMP into the organelle membrane, without ATP consumption (Diestelkötter & Just, 1993; Pinto *et al*, 2006). Interestingly, it was reported that the cytosolic domain of PEX3 has the capacity to oscillate between a lipid-free and lipid-bound state, a process that seems to be modulated by PEX19 (Pinto *et al*, 2009). This property is behind a model proposing that this domain triggers the dissociation of the PEX19-PMP complex and participates in the insertion of the PMP into the organelle membrane (Pinto *et al*, 2009). PEX16 is an integral membrane protein, whose function is still unknown. In mammalian cells, it was proposed it could function either as a receptor, providing the docking site at the peroxisomal membrane for PEX3-PEX19 complexes, or as a component of a putative membrane insertion machinery for PMPs (Kim *et al*, 2006; Matsuzaki & Fujiki, 2008). Surprisingly, although PEX16 is essential for mammalian peroxisome biogenesis, no PEX16 ortholog has been found in yeasts, with the exception of *Yarrowia lipolytica*. However, in *Y. lipolytica*, as well as in *Arabidopsis thaliana*, PEX16 seems to be implicated in functions other than membrane biogenesis (Guo *et al*, 2003; Lin, 1999; Kim & Mullen, 2013).

How the lipid components of the peroxisomal membrane are recruited to the peroxisomal membrane is still poorly understood, but considering that: 1) high resolution images obtained by electron microscopy demonstrate the existence of close physical interactions between peroxisomes and the ER (Rosenberger *et al*, 2009; Binns *et al*, 2006; Toulmay & Prinz, 2011; Elbaz & Schuldiner, 2011); 2) the peroxisomal membrane consists mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), resembling that of the ER (Fujiki *et al*, 1982); and 3) most of the enzymes involved in the synthesis of polar lipids are localized in the ER and not in peroxisomes (reviewed in (Gibellini & Smith, 2010)), it is commonly accepted that the ER provides the lipids for the formation of the peroxisomal

membrane. Two models of lipid trafficking have been proposed: 1) employment of specialized vesicles (Lazarow & Fujiki, 1985; Raychaudhuri & Prinz, 2008), or 2) direct transfer from the ER to peroxisomes at contact sites between both organelles (Raychaudhuri & Prinz, 2008). It should be noted that this last mechanism of lipid transfer has also been suggested to occur between peroxisomes and other organelles, such as lipid droplets or mitochondria (Rosenberger *et al*, 2009).

1.3.2. Peroxisome proliferation

Peroxisomes are highly dynamic organelles that have the remarkable capacity to adjust their number, size and enzyme content in response to developmental, environmental, and metabolic stimuli. In the last years several components of the peroxisome elongation and division machinery have been identified in mammals, yeast and plants (Fagarasanu *et al*, 2007; Lingard *et al*, 2008; Schrader & Fahimi, 2006; Thoms & Erdmann, 2005; Orth *et al*, 2007; Schrader, 1998; Passreiter *et al*, 1998; Erdmann & Blobel, 1995; Marshall *et al*, 1995).

Peroxisome proliferation in mammals, involves three isoforms of PEX11: PEX11α, PEX11β and PEX11γ (Li *et al*, 2002a, 2002b; Tanaka *et al*, 2003; Schrader, 1998). This conserved peroxin has a membrane remodeling activity, being able to deform and elongate the peroxisomal membrane (Lingard & Trelease, 2006; Li & Gould, 2002; Rottensteiner *et al*, 2003; Opaliński *et al*, 2011a, 2011b). It was observed that an N-terminal amphipathic helix of PEX11 interacts with liposomes that have a lipid composition resembling that of the peroxisomal membrane, inducing the membrane bending (Opaliński *et al*, 2011a). Along with this role in the reorganization of the peroxisomal membrane before fission (Schrader, 1998; Delille *et al*, 2010, 2011; Cepińska *et al*, 2011), PEX11 is also involved in the recruitment of the peroxisomal fission machinery (Li & Gould, 2003; Koch & Brocard, 2012).

In mammals, the scission machinery is shared with mitochondria (Koch *et al*, 2005; Schrader, 2006; Waterham *et al*, 2007; Gandre-Babbe & van der Bliek, 2008; Schrader *et al*, 2012) and includes two membrane adaptors, Fission1 (FIS1) and the mitochondrial fission factor (MFF) (Koch *et al*, 2005; Gandre-Babbe & van der Bliek, 2008; Itoyama *et al*, 2013), and the dynamin-like GTPase DLP1/Drp1 (Li & Gould,

2003; Koch *et al*, 2003, 2004; Tanaka *et al*, 2006). FIS1 and MFF potentially act as DLP1-recruiting factors to constriction sites on the peroxisomal membrane (Koch *et al*, 2005; Itoyama *et al*, 2013). DLP1 is a large and self-assembling GTPase that mediates peroxisome fission through the formation of ring-like structures around constricted membranes coupled with GTP hydrolysis (Smirnova *et al*, 2001; Praefcke & McMahon, 2004).

1.3.3. Import of peroxisomal matrix proteins

Presently, more than fifty different enzymes involved in the different peroxisomal metabolic pathways (see section 1.1.) have been identified in mammalian peroxisomes. Correct sorting of peroxisomal matrix proteins to the lumen of the organelle is ensured by one of two distinct peroxisomal targeting signals (PTS): a PTS1 or a PTS2 (Purdue & Lazarow, 2001). The vast majority of mammalian peroxisomal matrix proteins contain a PTS1 and only a small subset holds a PTS2 (Kunze *et al*, 2011). The PTS1 consists of a conserved tripeptide sequence located at the C terminus that was first identified in firefly luciferase as S-K-L (Gould *et al*, 1987). Later, this sequence was further extended to a broader consensus sequence that includes variants of the tripeptide [(S/A/C)-(K/R/H)-(L/M)] (Lametschwandtner *et al*, 1998). For the vast majority of PTS1-containing proteins, the tripeptide is sufficient for the proper targeting to the peroxisome. However, it was reported that, in some cases, other residues near the tripeptide are also involved (Brocard & Hartig, 2006; Lametschwandtner *et al*, 1998; Neuberger *et al*, 2003; Chowdhary *et al*, 2012; Lingner *et al*, 2011).

Only a few mammalian matrix proteins (three to four) harbor a PTS2 (Kunze *et al*, 2011). The number of PTS2 proteins in different organisms can vary a lot ranging from none in *Caenorhabditis elegans* (*C. elegans*) and in the diatome *Phaeodactylum tricornutum,* to a quarter of the total matrix proteins in *Arabidopsis thaliana* (*A. thaliana*) (Gonzalez *et al*, 2011; Gould *et al*, 1989; Kunze *et al*, 2011; Motley *et al*, 2000; Reumann *et al*, 2004, 2009; Lazarow, 2006). The PTS2 was first identified in rat liver thiolase as a conserved sequence at the N terminus of the protein (Swinkels *et al*, 1991; Osumi *et al*, 1991). Comparison between this thiolase

and several other PTS2-containing proteins resulted in the establishment of an N-terminal degenerated nonapeptide consensus sequence: R-(L/V/I/Q)-X_2-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A) (Petriv *et al*, 2004). Unlike the PTS1, which is not processed upon import, the PTS2 is cleaved in the peroxisomal matrix by proteolytic enzymes present in higher eukaryotes. These proteases have been recently identified in plants (DEG15) and mammals (Trypsin domain containing 1, TYSND1) (Lazarow, 2006; Helm *et al*, 2007; Mizuno *et al*, 2013; Kurochkin *et al*, 2007).

Newly synthesized peroxisomal matrix proteins are bound by specific cytosolic receptors that recognize their PTSs and escort them to the peroxisomal membrane. In mammals, plants and many other organisms, both PTS1 and PTS2 proteins are transported to the peroxisome by PEX5, the peroxisomal matrix protein shuttling receptor (Braverman *et al*, 1998; Dodt & Gould, 1996; Galland *et al*, 2007; Otera *et al*, 1998; Woodward & Bartel, 2005). The interaction between PEX5 and PTS1 proteins is direct (Figure 2) and is mediated by the PTS1 motif of the cargo protein on one hand, and the C-terminal tetratricopeptides repeats (TPRs) of PEX5 on the other, although other regions of both proteins are also involved (see also below) (Terlecky *et al*, 1995; Gatto *et al*, 2000; Klein *et al*, 2001, 2002; van der Klei & Veenhuis, 2006; Oshima *et al*, 2008; Freitas *et al*, 2011).

The interaction between PEX5 and PTS2 proteins is rather different (Figure 2), requiring the participation of another peroxin, PEX7 (Braverman *et al*, 1998; Otera *et al*, 1998; Matsumura *et al*, 2000; Woodward & Bartel, 2005; Lazarow, 2006; Galland *et al*, 2007). Based on a recent structure of a PEX18/PEX7/PTS2 complex, it is possible that PEX5 also interacts with the PTS2 directly (Pan *et al*, 2013). However, either this interaction is only possible in the presence of PEX7 (*e.g.*, due to conformational alterations) or is too weak to be productive, because PEX7 is absolutely essential for the targeting of these proteins to the organelle.

PEX7 presents six WD40 repeats which have the capacity to recognize and bind the PTS2 peptide (Marzioch *et al*, 1994; Zhang & Lazarow, 1996; Rehling *et al*, 1996; Elgersma *et al*, 1998). Unlike in mammals and plants, yeast and fungi PEX7 does not bind PEX5 and use instead species-specific receptors. These receptors are PEX18 and PEX21, two redundant proteins, in *S. cerevisiae*, and PEX20 in *Y.*

lipolytica, *Pichia pastoris*, *Hansenula polymorpha*, and *Neurospora crassa* (Titorenko *et al*, 1998; Purdue *et al*, 1998; Braverman *et al*, 1998; Sichting *et al*, 2003; Otzen *et al*, 2005; Woodward & Bartel, 2005; Léon *et al*, 2006; Lee *et al*, 2006; Schliebs & Kunau, 2006; Galland *et al*, 2007). All these species-specific peroxins display structural and functional similarities to the N-terminal half of the mammalian PEX5 which include the capacity to interact with PEX7 and PEX14 (see below) (Dodt *et al*, 2001; Einwächter *et al*, 2001; Nito *et al*, 2002; Lazarow, 2006; Schliebs & Kunau, 2006; Galland *et al*, 2007).

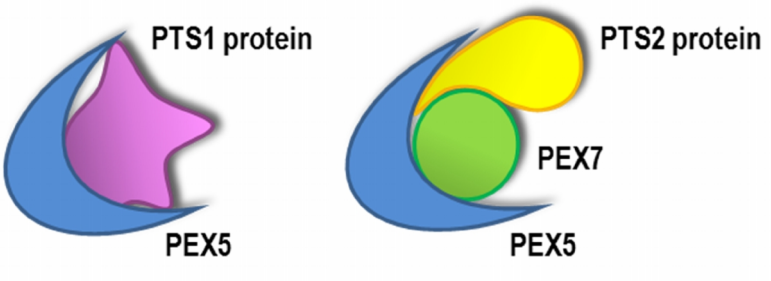


Figure 2. PEX5-mediated targeting of peroxisomal matrix proteins.
In mammals and plants, PEX5 is responsible for both PTS1 and PTS2 targeting. The interaction between PEX5 and matrix proteins containing a PTS1 is direct, while the PEX5-PTS2 interaction requires an adaptor protein, PEX7.

It should be pointed out that some peroxisomal matrix proteins do not possess an obvious PTS for PEX5 recognition but still depend on PEX5 for their targeting to organelle (Kragler *et al*, 1993; Peterson *et al*, 1997; Klein *et al*, 2002; Titorenko *et al*, 2002; Gunkel *et al*, 2004; Islinger *et al*, 2009; Galland & Michels, 2010). Different mechanisms to explain how these proteins reach the peroxisomal matrix have been proposed (van der Klei & Veenhuis, 2006). In the case of Acyl-CoA oxidase (ACOX1) from *S. cerevisiae* and *Y. lipolytica*, it was suggested the existence of an internal PTS within the protein, away from the carboxyl terminus, that interacts with the N-terminal half of PEX5 (Klein *et al*, 2002). The same reasoning has also been applied for some other non-PTS proteins (Gunkel *et al*, 2004; Oshima *et al*, 2008). Alternatively, it has been proposed that proteins lacking a PTS can reach the organelle by hijacking the peroxisomal sorting pathway, *i.e.*, by binding proteins that harbor a targeting signal. This is the so-called piggyback import mechanism (Glover *et al*, 1994; McNew & Goodman, 1994; van der Klei & Veenhuis, 2006; Islinger *et al*,

2009). It should be noted that this kind of mechanism has been considered because peroxisomes have the capacity to translocate co-factor bound, folded, and possibly even oligomerized proteins (McNew & Goodman, 1994; Walton *et al*, 1995; Lee *et al*, 1997; Titorenko *et al*, 2002). Nevertheless, the physiological relevance of this type of import mechanism is still poorly understood and, in fact, has been recently challenged (Freitas *et al*, 2011). Indeed, a recent study has shown that PEX5 binds newly synthesized matrix proteins which are oligomeric in their native state, strongly inhibiting their oligomerization. It was therefore proposed that PEX5 may be a chaperone/holdase, preventing premature protein interactions and keeping cargo proteins in a monomeric near-native conformation (Freitas *et al*, 2011). Such function is probably crucial to ensure that proteins that do not expose their PTS1 signals upon oligomerization can be efficiently targeted to the peroxisome (Freitas *et al*, 2011). However, if this is so, then there is no obvious reason to exclude a scenario where all newly synthesized peroxisomal matrix proteins are bound by PEX5 before they have the chance to oligomerize.

1.3.3.1. The peroxisomal matrix import receptor PEX5

PEX5 is a central peroxin in the mammalian peroxisomal matrix protein import pathway. As explained above it is responsible for the recognition and targeting of all matrix proteins to the organelle.

In mammals, PEX5 has two main isoforms that result from alternative splicing of the *PEX5* transcript: one with 639 amino acids and another with 602 amino acids. These are called the large and small isoforms of PEX5, respectively (Braverman *et al*, 1998). The difference between these two proteins is the presence of a PEX7-binding domain in the large isoform (between amino acids 214-215 of the small isoform) that allows targeting of PTS2 proteins to the peroxisome by PEX5 (Braverman *et al*, 1998; Otera *et al*, 1998; Dodt *et al*, 2001). PEX5 from yeasts and fungi lacks this PEX7-interacting domain and, consequently, does not transport PTS2 proteins (Purdue *et al*, 1998; Einwächter *et al*, 2001; Stein *et al*, 2002; Sichting *et al*, 2003). In plants, two scenarios can be found, with some species presenting only the larger mRNA of PEX5

(*e.g.,* in *A. thaliana*) and others the two mRNAs (*e.g.,* in *Oryza sativa*) (Hayashi *et al*, 2005; Woodward & Bartel, 2005; Lee *et al*, 2006).

Mammalian PEX5 is a 70-kDa protein that can be structurally and functionally divided into two domains: the N-terminal and the C-terminal halves (Costa-Rodrigues *et al*, 2005). Sequence alignment analysis of PEX5 from several species revealed a high degree of sequence conservation in the C-terminal domain of PEX5 in sharp contrast to its N-terminal half, which is poorly conserved with the exception of a few amino acids that comprise the multiple pentapeptide WXXXF/Y repeats, and a highly conserved cysteine residue that is present in the N terminus of all PEX5 proteins from all peroxisome-containing organisms (Kragler *et al*, 1998; Otera *et al*, 1998; Wimmer *et al*, 1998; Schliebs *et al*, 1999; Gatto & Geisbrecht, 2000; Lee *et al*, 2006; Bottger *et al*, 2000; Costa-Rodrigues *et al*, 2004).

As depicted in Figure 3, the C-terminal half of PEX5 is a structured domain that contains two clusters of three TPR motifs, TPRs 1-3 and TPRs 5-7, connected by a flexible hinge region (TPR4) (Terlecky *et al*, 1995; Gatto *et al*, 2000; Gatto & Geisbrecht, 2000; Klein *et al*, 2001). The pocket/groove formed between the two clusters of TPRs constitutes the PTS1-binding site (Gatto & Geisbrecht, 2000; Stanley *et al*, 2006). The TPR domain is flexible, alternating between an open “snail” conformation when unbound, and a locked “ring”-like conformation when interacting with a PTS1 (Stanley *et al*, 2006, 2007; Stanley & Wilmanns, 2006). The binding of PTS1 proteins by PEX5 TPRs is conserved between all species explaining why this domain is so highly conserved (Brocard *et al*, 1994; Nuttley *et al*, 1995; de Walque *et al*, 1999; Gatto & Geisbrecht, 2000; Terlecky *et al*, 1995; Klein *et al*, 2001).

Unlike its C-terminal region, the N-terminal half of PEX5 is a non-globular intrinsically disordered domain that harbors the binding motifs for other members of the Peroxisomal Import Machinery (PIM), such as PEX13 and PEX14, two components of the Docking Translocation Module (DTM), and, as mentioned before, PEX7 (Figure 3). In mammals, the diaromatic pentapeptide motifs [WXXX(F/Y)] of the N-terminal domain of PEX5 are required for the interactions with the components of the DTM (Azevedo & Schliebs, 2006; Neufeld *et al*, 2009; Saidowsky *et al*, 2001; Gouveia *et al*, 2000; Otera *et al*, 2002; Schliebs *et al*, 1999; Reguenga *et al*, 2001;

Bottger *et al*, 2000). The interaction between PEX5 and PEX14 involves multiple sites of interaction and it was demonstrated that each of the seven PEX5 diaromatic pentapeptides bind PEX14 with high affinity (with Kd values in the low nM range), thus resulting in a very strong interaction (Otera *et al*, 2002; Saidowsky *et al*, 2001; Schliebs *et al*, 1999). The PEX5-PEX13 interaction also requires the diaromatic motifs of PEX5 and the C-terminal SH3 motif of PEX13 both in mammals and yeasts but, in mammals, the N-terminal domain of PEX13 has also been suggested to have a role in this interaction (Otera *et al*, 2002; Bottger *et al*, 2000; Barnett *et al*, 2000; Williams & Distel, 2006; Costa-Rodrigues *et al*, 2005).

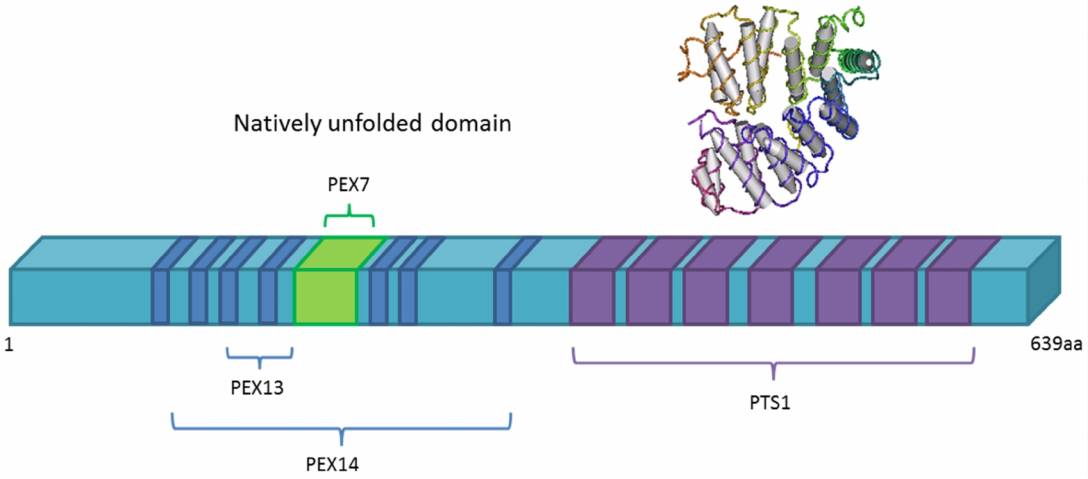


Figure 3. Structure of the large isoform of mammalian PEX5.
Schematic diagram of the mammalian PEX5 large isoform structure with the interaction sites for the components of the PIM. The dark blue bars represent the diaromatic motifs (WxxxF/Y; PEX14 and PEX13 binding sites). The green box indicate the extra 37 amino acids present in the larger isoform of PEX5 that harbor the PEX7 binding site (absent in the small isoform of PEX5). The purple boxes represent the TPR motifs that recognize and bind the PTS1. A crystal structure of these motifs is represented above (Protein Data Bank accession number 1FCH). Abbreviations: aa, amino acids; TPR, tetratricopeptide repeats.

Although size exclusion chromatography (SEC) analyses suggested that PEX5 might be a tetramer, with an apparent molecular mass of approximately 270 kDa, biophysical and biochemical independent approaches demonstrated that PEX5 is actually monomeric in solution, and that the abnormally large Stokes radius determined by SEC analyses is due to the natively unfolded pre-molten globule-like character of the N-terminal domain of PEX5 (Schliebs *et al*, 1999; Costa-Rodrigues *et al*, 2005; Carvalho *et al*, 2006).

1.3.3.2. The PEX5-mediated peroxisomal protein import pathway

The fact that PEX5 displays *in vivo* a dual subcellular localization (being predominantly cytosolic but also peroxisomal) along with its capacity to recognize and bind cargo proteins, led to the hypothesis that PEX5 is a shuttling receptor for peroxisomal matrix proteins (McCollum *et al*, 1993; Van der Leij *et al*, 1993; Brocard *et al*, 1994; Dodt *et al*, 1995; Wiemer *et al*, 1995; Fransen *et al*, 1995; Nuttley *et al*, 1995; van der Klei *et al*, 1995; Dodt & Gould, 1996; Elgersma *et al*, 1996; Wimmer *et al*, 1998; de Walque *et al*, 1999; Jardim *et al*, 2000). Evidence for this hypothesis came from the observation that the subcellular distribution of PEX5 can be manipulated in live cells just by decreasing the temperature of the cell culture or the intracellular concentration of ATP (Dodt & Gould, 1996). Under these conditions, the fraction of PEX5 localizing at peroxisomes increases, a situation that can be reverted by returning the cells to normal conditions. Apparently, PEX5 can undergo multiple cycles of association and dissociation with the peroxisome.

Our understanding on the PEX5-mediated protein import pathway has increased dramatically over the last years. Data from protein complex analyses (such as immunoprecipitations, Blue Native PAGE and sucrose gradients), cell biology experiments and *in vitro* import assays have contributed for a more precise and detailed clarification of how the PIM operates and on the architecture of its components (Agne *et al*, 2003; Albertini *et al*, 2001; Reguenga *et al*, 2001; Collins *et al*, 2000a; Gouveia *et al*, 2000). So far, all the gathered data points to a dynamic architecture of the PIM, where several steps of the pathway take place in a large protein complex in which PEX5 is transiently integrated.

The mammalian PIM comprises ten mammalian peroxins, most of which are part of one of two functional/structural modules: 1) the DTM, *i.e.*, a membrane-embedded multisubunit complex that comprises PEX13, PEX14, and the “Really Interesting New Gene” (RING) peroxins PEX2, PEX10, and PEX12 (Agne *et al*, 2003; Reguenga *et al*, 2001; Oeljeklaus *et al*, 2012); and 2) the Receptor Export Module (REM), composed by the two peroxisomal “ATPases associated with diverse cellular activities” (AAA ATPases), PEX1 and PEX6, together with their peroxisomal membrane anchor, PEX26 (Matsumoto *et al*, 2003; Fujiki *et al*, 2012a) (see Figure 4).

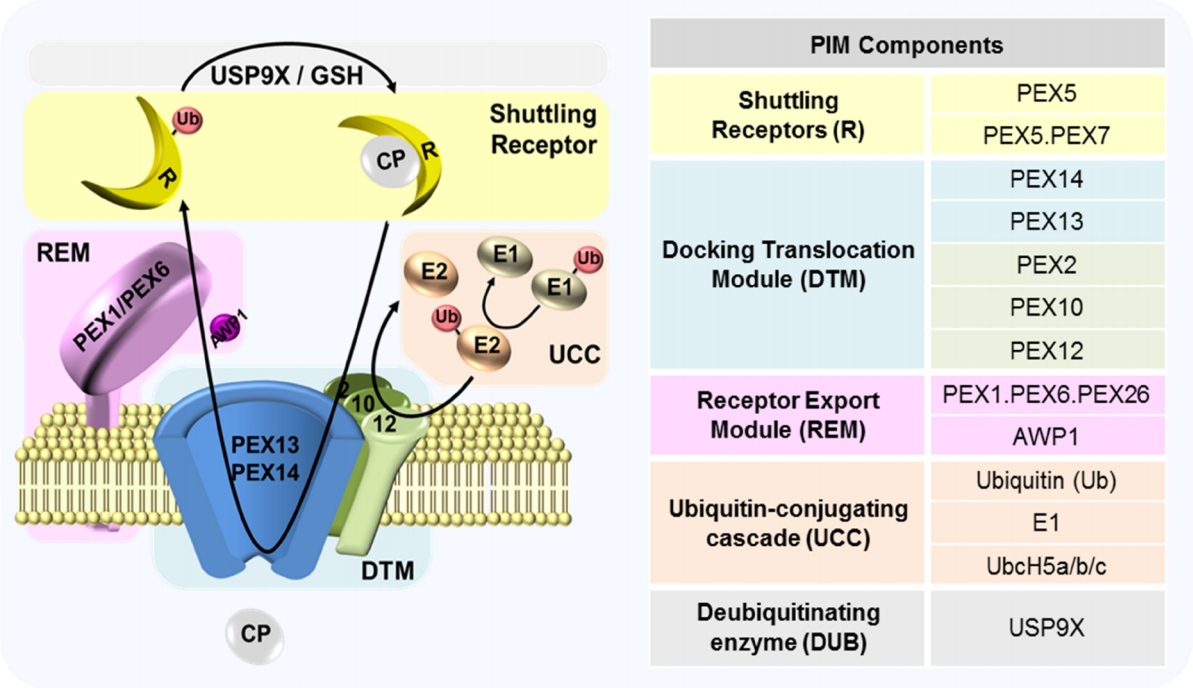
Finally, the cytosolic receptors, PEX5 and PEX7, complete the list of peroxins involved in the delivery of newly synthesized proteins to the organelle lumen (Dodt *et al*, 1995; Fransen *et al*, 1995; Braverman *et al*, 1997). Besides peroxins, the PIM also includes other proteins involved in ubiquitination/deubiquitination events occurring in this import pathway. Due to their involvement in other cellular pathways these proteins are not considered peroxins (Grou *et al*, 2008, 2012; Miyata *et al*, 2012).

Using a PEX5-centered *in vitro* import system, our laboratory has provided valuable data regarding the mechanistic details of the mammalian PEX5-mediated protein import pathway. In this system, an organelle suspension is incubated with radiolabeled PEX5 under different experimental conditions and the behavior of the radiolabeled protein is monitored by a protease-protection assay. The model presented in Figure 5 harbors all the data gathered so far regarding this import pathway.

After binding newly synthesized matrix proteins in the cytosol, PEX5 targets them to the peroxisomal membrane, where it interacts with members of the DTM. PEX13 and PEX14 are two integral membrane components of the DTM that have been shown to interact with the shuttling receptors PEX5 and PEX7, but not with the cargo proteins, suggesting that they serve as docking sites at the peroxisomal membrane for the PEX5-cargo complex (Girzalsky *et al*, 1999; Urquhart *et al*, 2000; Bottger *et al*, 2000; Shimizu *et al*, 1999; Fransen *et al*, 1998; Otera *et al*, 2002; Azevedo & Schliebs, 2006). As it is further discussed, a role in the cargo translocation step has also been proposed for PEX14 (Gouveia *et al*, 2000; Oliveira *et al*, 2003; Azevedo & Schliebs, 2006).

After docking at the DTM, PEX5 gets inserted into this protein machinery in a cargo- and temperature-dependent manner (Costa-Rodrigues *et al*, 2004; Gouveia *et al*, 2003b). At this stage, PEX5 adopts a transmembrane topology exposing a 2-kDa N-terminal domain into the cytosol while the bulky part of its polypeptide chain faces the organelle lumen (Gouveia *et al*, 2003a, 2000). Remarkably, insertion of PEX5 into the DTM does not require the hydrolysis of cytosolic ATP, a finding that led to the proposal that the strong protein-protein interactions established by PEX5 on one hand, and the DTM on the other, are the driving force for the insertion of the receptor-

cargo complex into the peroxisomal membrane and, perhaps, for the translocation of the cargo into the organelle matrix (Azevedo *et al*, 2004; Grou *et al*, 2009a; Oliveira *et al*, 2003). The observation that the N-terminal domain of PEX14, the region that interacts with the diaromatic motifs of PEX5, is either deeply embedded in the peroxisomal membrane or even exposed to the peroxisomal matrix, strongly supports this idea (Saidowsky *et al*, 2001; Oliveira *et al*, 2002).



PIM Components	
Shuttling Receptors (R)	PEX5
	PEX5.PEX7
Docking Translocation Module (DTM)	PEX14
	PEX13
	PEX2
	PEX10
	PEX12
Receptor Export Module (REM)	PEX1.PEX6.PEX26
	AWP1
Ubiquitin-conjugating cascade (UCC)	Ubiquitin (Ub)
	E1
	UbcH5a/b/c
Deubiquitinating enzyme (DUB)	USP9X

Figure 4. The mammalian peroxisomal import machinery (PIM).
Schematic view of the mammalian import machinery. The components of the PIM can be organized according to their functions. At the peroxisomal membrane, there are two major multisubunit protein complexes that belong to the PIM: the Docking/Translocation Module (DTM) that comprises PEX13, PEX14 and the RING peroxins PEX2, PEX10 and PEX12, and the Receptor Export Module (REM) that is composed by PEX1 and PEX6, the two peroxisomal AAA ATPases, and their membrane anchor, PEX26. The shuttling receptors (R), AWP1, elements of the ubiquitin-conjugating cascade (UCC; ubiquitin (Ub), the Ub-activating enzyme (E1) and the Ub-conjugating enzyme (E2; UbcH5a/b/c)) and the deubiquitinating enzyme (DUB; USP9X), complete the list of proteins that constitute the PIM. CP, cargo protein.

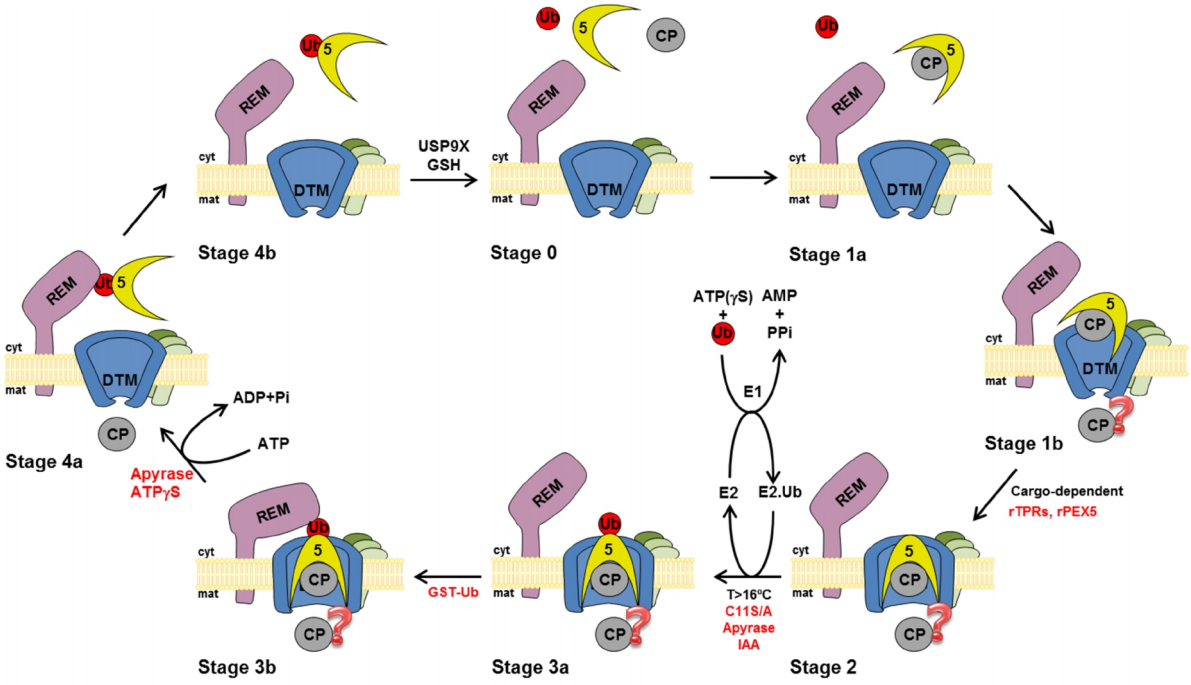


Figure 5. The PEX5-mediated protein import pathway.

Using a PEX5-centered *in vitro* import system, it was possible to detect and characterize five different PEX5 populations referred to as stage 0-4, respectively (substages a and b are essentially of conceptual nature). Several strategies/tools to block this pathway at different steps have been developed (shown in red) and are also indicated in the figure. Stage 0 – PEX5 is soluble and cargo-free (PEX5 is completely accessible to an exogenously added protease); Stage 1 – Cytosol-exposed PEX5-cargo complex (PEX5 is completely accessible to an exogenously added protease); Stage 2 – PEX5-cargo complex gets inserted into the DTM with PEX5 exposing the N terminus to the cytosol and most of its polypeptide chain to the organelle lumen (only 2 kDa of the N-terminal domain of PEX5 are accessible to protease treatment); Stage 3 – DTM embedded PEX5 is monoubiquitinated (PEX5 is monoubiquitinated and completely resistant to protease treatment); Stage 4 – Monoubiquitinated DTM-embedded PEX5 is removed from the membrane back to the cytosol, in an ATP-dependent manner, by the two mechanoenzymes, PEX1 and PEX6 (REM) (at this stage, monoubiquitinated PEX5 is completely protease accessible); Transition of stage 4 to stage 0, *i.e.*, the deubiquitination of soluble monoubiquitinated PEX5 to restore free soluble PEX5 is achieved by enzymatic (USP9X) and non-enzymatic (GSH) mechanisms. The step at which the cargo is translocated and released to the peroxisome matrix is unknown, but it has been suggested to occur between stage 1 and 2, with PEX5 pushing the cargo across the membrane as it gets inserted into the membrane (Gouveia *et al*, 2003a; Oliveira *et al*, 2003; Azevedo *et al*, 2004). Abbreviations used: 5, PEX5; CP, cargo protein; Ub, ubiquitin; DTM, Docking/Translocation Module; REM, Receptor Export Module; cyt, cytosol; mat, matrix; rTPRs, recombinant protein comprising only the PEX5 tetratricopeptide repeats domain; rPEX5, recombinant PEX5; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; IAA, iodoacetamide; C11S/A, PEX5 protein mutated version with the conserved cysteine 11 replaced by a serine or an alanine residue; GSH, glutathione.

The interactions established between the DTM and the DTM-embedded PEX5 are essentially irreversible (Costa-Rodrigues *et al*, 2004), therefore it is not surprising that the removal of PEX5 from the peroxisomal membrane back to the cytosol requires energy input. Surprisingly, this energy-dependent process is preceded by monoubiquitination of PEX5 at a conserved N-terminal cysteine residue[1] (position 6 and 11 in yeast and mammals, respectively) (Williams *et al*, 2007; Carvalho *et al*, 2007b). This modification is mandatory for the subsequent step of the pathway, the ATP-dependent PEX5 removal from the peroxisomal membrane back into the cytosol, a process catalyzed by the REM (Oliveira *et al*, 2003; Carvalho *et al*, 2007b; Platta *et al*, 2007). These findings provided the explanation for earlier reports showing that deletion of the first 17 amino acids containing this conserved cysteine residue as well as its alkylation or mutation to a serine residue, result in PEX5 proteins still able to enter the DTM, but that are no longer substrates for the REM (Costa-Rodrigues *et al*, 2004; Carvalho *et al*, 2007b, 2006).

The finding that mammalian PEX5, like its yeast counterpart, is monoubiquitinated at the DTM was unexpected at the time because, as stated before (see section 1.3.), mammals lack PEX4 and PEX22. On one hand, it was now obvious that the PIM of yeasts and mammals operate using similar principles, despite significant differences in their protein composition (see Table 4 in section 1.3.). On the other hand, it was evident that we were still missing components of the mammalian PIM. Using a peroxisome-dependent PEX5 monoubiquitination assay it was found that the long-sought mammalian E2 activity co-fractionated with cytosolic proteins (Grou *et al*, 2008). In fact, a simple low-speed centrifugation of a postnuclear supernatant (PNS) was sufficient to separate peroxisomes from the E2 activity involved in this ubiquitination event indicating that, contrary to yeasts/fungi and probably plants (Wiebel & Kunau, 1992; Koller *et al*, 1999; Zolman *et al*, 2005), mammalian peroxisomes do not contain a peroxisomal dedicated E2. Purification of the factor promoting this activity by standard protein purification procedures led to the

[1] Note that although ATP is surely needed to activate ubiquitin, we do not consider that the energy involved in monoubiquitination of PEX5 contributes for its export because the ΔG of the corresponding transthiolation reaction should be close to 0 kcal/mol (Grou *et al*, 2009b).

identification of the multipurpose cytosolic rat E2s, E2D1/2/3 (UbcH5a/b/c in humans) (Grou *et al*, 2008; Gonen *et al*, 1999; Saville *et al*, 2004).

Considering that three of the five core components of the DTM contain RING Zn^{2+}-binding domains, which define the largest class of E3 ubiquitin ligases, it was evident from the very first findings on receptor ubiquitination that these three peroxins, PEX2, PEX10 and PEX12, should have a role in this event (Liu & Subramani, 2013; Williams *et al*, 2008; Kaur *et al*, 2013; Platta *et al*, 2009). In line with this hypothesis, it was reported that the Zn^{2+}-binding domains of these peroxins have E3 activity in *in vitro* ubiquitination assays (Williams *et al*, 2008; Platta *et al*, 2009; Kaur *et al*, 2013). Noteworthy, several studies reported that PEX5 can still enter the DTM in cells lacking these peroxins (Dodt & Gould, 1996; Chang *et al*, 1999; Collins *et al*, 2000b; Agne *et al*, 2003). Thus, the DTM resembles a multisubunit E3 ligases (*e.g.*, cullin RING ligases) in which substrates are recruited not by the RING proteins but by other subunits of the protein complex (Grou *et al*, 2009a; Francisco *et al*, 2013).

The mechanistic details of how monoubiquitinated PEX5 is recognized by the REM are still not entirely understood. In principle, the REM could interact directly with DTM-embedded monoubiquitinated PEX5 or, alternatively, the recognition could be mediated by an ubiquitin-binding adaptor protein. Some data supporting this last possibility was recently reported. Indeed, AWP1 was identified as a protein possibly involved in the interaction between monoubiquitinated PEX5 and the REM (Miyata *et al*, 2012). After dislocation of DTM-embedded monoubiquitinated PEX5 into the cytosol, PEX5 is deubiquitinated to yield free soluble PEX5. This step probably occurs very fast *in vivo* because so far soluble thiol-sensitive monoubiquitinated PEX5 species were never detected (Grou *et al*, 2009b; Williams *et al*, 2007). Using an unbiased biochemical approach, USP9X was identified as the most active mammalian deubiquitinase (DUB) acting on monoubiquitinated PEX5 (Grou *et al*, 2012). Interestingly, however, knockdown of USP9X did not result in the cytosolic accumulation of monoubiquitinated PEX5 as it would be expected if this DUB were the only factor capable of deubiquitinating PEX5. Three possibilities may explain this result: 1) the low levels of USP9X still present in the knockdown experiments may be

sufficient to catalyze PEX5 deubiquitination; 2) less active/redundant DUBs may deubiquitinate PEX5 in the absence of USP9X; or 3) non-enzymatic mechanisms may also be involved in the deubiquitination of PEX5 (Grou *et al*, 2012). Indeed, it was demonstrated that physiological concentrations of glutathione are sufficient to disrupt the labile thioester bond linking ubiquitin to PEX5 in only a few minutes (Grou *et al*, 2009b). Interestingly, DTM-embedded monoubiquitinated PEX5 is protected from both enzymatic and non-enzymatic deubiquitination mechanisms, thus preventing futile ubiquitination/deubiquitination cycles at the DTM (Grou *et al*, 2009b).

Most of the mechanistic data presented above for the PEX5-mediated import pathway, was obtained using a PEX5-centered *in vitro* import system. However, this PEX5-centered model lacks important information concerning the cargo protein, namely on how and when it is translocated across the peroxisomal membrane. Two different scenarios have been proposed: 1) the driving force for the protein translocation could reside in the strong protein-protein interactions established between PEX5 and components of the DTM, with the translocation of the cargo occurring as PEX5 gets inserted into the DTM, with no ATP consumption (Gouveia *et al*, 2003a; Oliveira *et al*, 2003; Azevedo *et al*, 2004); and 2) cargo translocation and release could be linked to the ubiquitination and export of PEX5 back to the cytosol, coupling the energy requirements of the receptor monoubiquitination/extraction with cargo translocation (Williams *et al*, 2007; Grou *et al*, 2009a; Schliebs *et al*, 2010). Recently, in an attempt to answer these questions, a PTS2-centered *in vitro* import system was developed in our laboratory (Alencastre *et al*, 2009). The data obtained suggests that translocation of pre-thiolase, one of the 3-4 PTS2 proteins in mammals, across the peroxisomal membrane occurs before ubiquitination of DTM-embedded PEX5, probably while the receptor gets inserted into the DTM (Alencastre *et al*, 2009). However, considering that mammalian PTS2 proteins are targeted to the peroxisome lumen with the help of the adaptor protein PEX7, and that there are some data suggesting that this peroxin may enter the organelle matrix together with the PTS2 proteins, it remains unclear whether the mechanistic data gathered for PTS2 proteins is a general property of the peroxisomal protein import machinery or a unique feature of the PTS2 import pathway (Swinkels *et al*, 1991; Kunze *et al*, 2011;

Lazarow, 2006; Braverman *et al*, 1997, 1998; Otera *et al*, 1998; Nair *et al*, 2004). The main aim of the work presented here was to answer this question.

2. AIMS

AIMS

Our laboratory has been using an *in vitro* import strategy to dissect the mechanism of protein translocation across the mammalian peroxisomal membrane. Currently, our knowledge on the pathway followed by PEX5 during the protein import process is reasonably detailed. However, the same cannot be stated regarding the cargo proteins themselves. In an attempt to fill in this gap, the import mechanism of a PTS2 protein has been recently characterized. It was proposed that translocation of this protein across the peroxisomal membrane occurs upstream of PEX5 monoubiquitination. Yet, it is still unknown whether this property is a particularity of the PTS2 sorting pathway or a general property of the peroxisomal protein import machinery.

The main aim of this work was the development of an *in vitro* import system centered on a PTS1 cargo protein so that we could: 1) identify the step(s) at which the PTS1 cargo protein is moved from the cytosolic side of the peroxisomal membrane into the DTM; 2) determine the timing of the PTS1 cargo protein release from the DTM into the peroxisomal matrix; and 3) define the energetics of the protein translocation process.

3. EXPERIMENTAL PROCEDURES

3.1. Expression and purification of recombinant proteins

The recombinant large isoform of human PEX5 [hereafter simply referred to as PEX5; (Fransen *et al*, 1998; Braverman *et al*, 1998; Costa-Rodrigues *et al*, 2005)], proteins comprising amino acid residues 1-324 and 315-639 of PEX5 [ΔC1PEX5 and TPRs, respectively; (Carvalho *et al*, 2006)], PEX5 containing the missense mutation N526K [PEX5(N526K), (Gatto & Geisbrecht, 2000; Dodt *et al*, 1995; Carvalho *et al*, 2007a)], TPRs with the missense mutation N526K [TPRs(N526K), numbering of full-length PEX5; (Carvalho *et al*, 2007a)], a protein comprising the first 80 amino acid residues of human PEX14 [NDPEX14; (Carvalho *et al*, 2006)], PEX19 (Pinto *et al*, 2006) and a Glutathione S-Transferase-Ubiquitin fusion protein [GST-Ub; (Carvalho *et al*, 2007b)] were obtained as previously described.

A mutated PEX5 version possessing an alanine at position 11 was obtained with the QuikChange® Site-Directed Mutagenesis kit (Stratagene), using pQE30-PEX5 as the template (Costa-Rodrigues *et al*, 2005) and the primers listed in Table 5 (section 3.10.) previously described (Grou *et al*, 2009b). Expression in the M15 strain of *Escherichia coli* and purification with HIS-Select™ Nickel Affinity Gel (Sigma) were performed also as described before (Carvalho *et al*, 2006).

3.2. Plasmids and synthesis of radiolabeled proteins

The cloning strategy for PEX5 mutated versions possessing a lysine or alanine residue at position 11 [PEX5(C11K) or PEX5(C11A), respectively] were described elsewhere (Grou *et al*, 2009b).

The cDNAs encoding full-length mouse Sterol Carrier Protein x [SCPx; clone MmCD00313611, PlasmID, Dana Farber/Harvard Cancer Center; (Seedorf *et al*, 1993)] and full-length human 2,4-Dienoyl-CoA Reductase [DECR2; plasmid pKDN36, kindly provided by Dr. Marc Fransen (De Nys *et al*, 2001)] were amplified by PCR using the primers listed in Table 5, section 3.10. The amplified sequences were subsequently digested with XbaI and KpnI and cloned into the XbaI/KpnI-digested pGEM4® vector (Promega), originating pGEM4-SCPx and pGEM4-DECR2, respectively.

^{35}S-Labeled proteins were synthesized using the TNT® T7 Quick Coupled Transcription/Translation System (Promega) in the presence of [^{35}S]methionine (specific activity >1000 Ci/mmol; PerkinElmer Life Sciences) following the standard conditions of the manufacturer.

3.3. Preparation of postnuclear supernatants (PNS) from mouse liver

Mouse liver PNS fractions for *in vitro* assays were prepared from male C57BL/6J mice with approximately 6 weeks of age. Mice were fasted overnight and euthanized by cervical dislocation. The livers were quickly removed, minced and homogenized in ice-cold SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 8.0) supplemented with 2 µg/ml N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64). After centrifuging the homogenates twice at 600 x g for 10 min at 4 ºC (SS-34 rotor in a RC5B Sorvall® centrifuge), the resulting supernatant was aliquoted, frozen in liquid nitrogen and stored at -70 ºC. Protein content of PNS fractions was determined using the Bradford method and rabbit immunoglobulins as protein standards.

3.4. *In vitro* import experiments:

3.4.1. ^{35}S-Scpx *in vitro* peroxisomal import assays

In a standard import reaction, 400 µg of mouse liver PNS protein (per 100 µl reaction) were primed for import by incubation in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.2, 3 mM $MgCl_2$, 20 µM methionine, 2 µg/ml E-64) supplemented with 0.3 mM ATP for 5 min at 37 ºC, as described previously (Oliveira *et al*, 2003). One microliter of the reticulocyte lysate containing ^{35}S-labeled SCPx was diluted 1:10 in import buffer and pre-incubated for 15 min at 37 ºC in the presence of PEX5 (30 nM final concentration). Ten microliters of the diluted lysate were subsequently added to the PNS in import buffer supplemented with 2 mM glutathione (GSH), 3 mM ATP and 15 µM of a bovine ubiquitin. The import reaction was allowed to proceed for 15 min at 37 ºC and, after import, samples were digested with 400 µg/ml of Proteinase K (PK) for 40 min on ice. Protease was inactivated with 500 µg/ml

of phenylmethylsulfonyl fluoride (PMSF) and incubated for 3 min on ice. The organelle suspensions were diluted to 1 ml with SEMK (SEM buffer containing 80 mM KCl) and organelles were isolated by centrifugation (16,000 x g, 20 min, 4 ºC). Protein in the samples was precipitated with 10% (w/v) trichloroacetic acid (TCA) for 30 min on ice. After centrifugation (16,000 x g, 15 min, 4 ºC), the precipitated protein was washed with acetone, subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were stained with Ponceau S, exposed to an x-ray film and afterwards probed with the relevant antibodies.

When specified, the standard protocol was modified and the following alterations were performed:

1) In experiments aiming at analyzing the effect of pre-incubating SCPx with recombinant PEX5 in its import efficiency, the reticulocyte lysate containing ^{35}S-SCPx was also diluted in import buffer and pre-incubated for 15 min at 37 ºC in the absence of PEX5. After the addition of the diluted lysate to a PNS, the same amount of recombinant PEX5 (30 nM, see above) was added, and the import reaction performed under the same conditions.

2) In experiments evaluating the specificity of the *in vitro* import system using different recombinant proteins, the pre-incubation step was altered: one microliter of the reticulocyte lysate containing ^{35}S-labeled SCPx was diluted 1:10 in import buffer and pre-incubated for 15 min at 37 ºC in the presence of one or more of the following recombinant proteins: PEX5 or PEX5(N526K) or PEX5(C11A) (30 nM final concentration), TPRs or TPRs(N526K) (10 µM final concentration), and NDPEX14 or PEX19 (20 µM final concentration).

3) When evaluating the energetic requirements of ^{35}S-SCPx import, the diluted lysate added to the PNS was supplemented with 2 mM glutathione and, where indicated, ATP (3 mM) or AMP-PNP (3 mM), and bovine ubiquitin (15 µM). In the apyrase experiments, both the diluted lysate and the PNS in import buffer were incubated at 37 ºC with apyrase (20 units/ml, Grade VII, Sigma) for 5 and 2 min, respectively, before starting the import assay. The import reaction was incubated for

just 7 min to minimize differences induced by the time-dependent occupation of the DTM by PEX5 (Alencastre *et al*, 2009).

3.4.2. ^{35}S-PEX5 *in vitro* import assays

Control experiments for the AMP-PNP and apyrase treatments in the PEX5-mediated import pathway were performed as follows. For the AMP-PNP experiment, the reticulocyte lysate containing ^{35}S-labeled PEX5(C11K) was diluted 1:10 in import buffer and added to 400 µg of PNS protein that had been primed for import. Import assays contained 2 mM of GSH, 15 µM of bovine ubiquitin, 3 µM of ubiquitin aldehyde (Ubal) and either 3 mM of ATP or AMP-PNP. After 7 min at 37 ºC, the import reactions were centrifuged to separate organelles from the soluble phase. Both fractions were analyzed by SDS-PAGE/Western-blotting. In the apyrase assay, the reticulocyte lysate containing ^{35}S-labeled PEX5(C11K) was diluted 1:10 in import buffer and halved. Half of the sample was incubated in the absence of apyrase and added to a primed PNS in ATP-containing import buffer. The second half of the diluted lysate was treated with apyrase (20 units/ml final concentration, for 5 min at 37 ºC) and added to an apyrase-treated PNS (20 units/ml final concentration, for 2 min at 37 ºC). Import reactions were supplemented with 2 mM of GSH and 15 µM of GST-Ub and incubated at 37 ºC for 7 min. The organelles were then isolated by centrifugation and samples were processed as described above.

3.5. Sucrose gradient centrifugation analyses of proteins

For the sucrose gradient centrifugation analyses, 10 µl of SCPx lysate were incubated with 25 µg of recombinant PEX5 in 200 µl of a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA-NaOH, pH 8.0, and 1 mM DTT or with buffer alone. After incubation for 10 min at room temperature, 30 µg of each of the following internal sedimentation coefficient standards were added to the samples: ovalbumin (3.6 S), bovine serum albumin (4.3 S) and aldolase (7.4 S). Protein mixtures were then loaded onto the top of a continuous 5-20% (w/v) sucrose gradient in the same buffer. After centrifugation at 247,000 x g for 29 h at 4 °C in a SW41 swing-out rotor (Beckman), 13 equal fractions were collected from the bottom of the

gradient. One hundred and fifty microliters of each fraction were precipitated with TCA precipitation and analyzed by SDS-PAGE as described above.

3.6. ^{35}S-SCPx susceptibility to Proteinase K (PK) digestion

To evaluate ^{35}S-SCPx susceptibility to PK digestion several experiments were performed:

1) One microliter of the reticulocyte lysate containing ^{35}S-labeled SCPx was diluted 1:10 in import buffer and pre-incubated for 15 min at 37 ºC in the absence or presence of PEX5 (30 nM final concentration). Both samples were then treated with 400 µg/ml of PK and incubated on ice for 40 min. After protease inactivation, samples were precipitated with 10% (w/v) TCA.

2) Five microliters of the reticulocyte lysate containing ^{35}S-SCPx were mixed with 2 mg of PNS in import buffer containing or not 1% (w/v) Triton X-100 (TX-100). Aliquots containing 400 µg of PNS were subsequently treated with different amounts of PK (0 to 500 µg/ml). Protease was inactivated with 500 µg/ml of PMSF and incubated for 3 min on ice. Samples were subsequently precipitated with 10% (w/v) TCA.

3) After a standard *in vitro* import reaction, organelles were isolated by centrifugation, resuspended in import buffer and subjected to PK digestion (50 µg/ml), in the absence or presence of 1% (w/v) TX-100. After protease inactivation with 50 µg/ml of PMSF, samples were subjected to TCA precipitation.

3.7. Sub-fractionation of peroxisomes

Sub-fractionation of peroxisome proteins was performed as follows. Protease-treated organelles were isolated by centrifugation and resuspended in 1 ml of SEM buffer supplemented with 1:500 (v/v) PMSF and mammalian protease inhibitor cocktail (Sigma), and 2 mM DTT. After sonication, half of the sample was kept on ice, while the other half was separated into membrane and soluble fractions by centrifugation for 45 min at 135,000 x g. Samples were subjected to precipitation with

10% (w/v) of TCA and subjected to SDS-PAGE/Western-blotting. After exposing to an X-ray film, membranes were probed with several antibodies.

3.8. Density gradient centrifugation of import reactions

For the Nycodenz gradient centrifugation analysis of import reactions, a 4-fold scale-up of the standard import reaction was used. After PK treatment, the complete import mixture was diluted to 1.5 ml with SEM buffer and applied onto a Nycodenz step gradient. The Nycodenz step gradient was used as previously described for rat liver with minor modifications (Pinto *et al*, 2006). The Nycodenz step gradient consisted in 4 steps: 1.5 ml of 45% (w/v), 7 ml of 28% (w/v), 2 ml of 25% (w/v), and 2 ml of 20% (w/v) Nycodenz in 5 mM MOPS-KOH, pH 7.2, and 1 mM EDTA-NaOH, pH 8.0. After centrifugation at 59,000 x g for 3 h at 4 ºC in a vertical rotor (STEPSAVER^{TM} 65V13, Sorvall®), 14 equal fractions were collected from the bottom of the gradient. Two hundred and fifty microliters of each fraction were precipitated with TCA, subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S and exposed to an x-ray film. Afterwards, it was probed with the relevant antibodies.

3.9. Antibodies

The antibodies directed to SCPx (19182-1-AP; ProteinTechTM), catalase (C0979; Sigma), KDEL (ab12223; Abcam), and cytochrome c (556433; BD PharmingenTM) were purchased. The antibody directed to PEX14 was described before (Reguenga *et al*, 2001). Rabbit and mouse antibodies were detected using goat alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies (A9919 and A2429, respectively; Sigma) or goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology).

3.10. Primers list

Table. 5. List of oligonucleotides used for cloning in this work

construct	Primers	Sequence
pQE30-PEX5(C11A)	P5C11A Fw	5'–GGTGGAGGCCGAAGCCGGGGGTGCCAAC–3'
	P5C11A Rv	5'–GTTGGCACCCCCGGCTTCGGCCTCCACC–3'
pGEM4-SCPx	SCPx_Fw	5'–GCGCCGTCTAGAGCCACCATGCCTTCTGTCGCTTTG–3'
	SCPx_Rev	5'–GCCGGCGGTACCTCACAGCTTAGCTTTGCCC–3'
pGEM4-DECR2	DECR2_Fw	5'–GATAATTCTAGAGCCACCATGGCCCAGCCGC–3'
	DECR2_Rev	5'–CGCCGTGGTACCCTAGAGCTTAGCAGAGAAGGA–3'

4. RESULTS

4.1. Development of an *in vitro* import system centered on a PTS1 protein.

An *in vitro* import system has been used in our laboratory to study how the peroxisomal import machinery operates. Experimentally this *in vitro* strategy comprises three steps. First, an *in vitro* transcription/translation kit, usually a rabbit reticulocyte lysate, is used to synthesize a ^{35}S-labeled reporter protein. In the second step, the reporter protein is incubated under appropriate experimental conditions with a PNS, *i.e.*, a subcellular fraction that contains peroxisomes and all the cytosolic components involved in the PEX5-mediated protein import pathway. Finally, the organelle suspension is subjected to a protease-protection assay, *i.e.*, the import reaction is treated with a large amount of a non-specific protease (*e.g.*, proteinase K) to degrade the fraction of non-imported (thus accessible) reporter protein while preserving the fraction that was imported into the organelles. While this strategy works quite well when the reporter protein is PEX5 itself (Gouveia *et al*, 2003a; Grou *et al*, 2009b; Miyata & Fujiki, 2005), the import yields obtained using peroxisomal matrix proteins as reporters are generally poor. As explained in a previous work (Alencastre *et al*, 2009), a major limitation rises from the fact that PNS contains large amounts of soluble PTS1 proteins that have leaked from peroxisomes during tissue homogenization (Imanaka *et al*, 1987; Alexson *et al*, 1985). Since the PTS1 is not cleaved upon import (Purdue & Lazarow, 2001), these soluble proteins compete with the ^{35}S-labeled reporter protein for PEX5 binding, thus resulting in low import yields. Addition of recombinant PEX5 to *in vitro* import reactions to minimize this competition problem results in import yields that are still modest (see below; and unpublished results). Probably, the addition of the recombinant protein to the import reaction results in the increase of complexes comprising PEX5 and endogenous PTS1 proteins, producing a new competition problem at the DTM.

To overcome these problems, a new strategy was developed: the incubation of a radiolabeled PTS1 protein with recombinant PEX5 prior to the *in vitro* import reaction. Two main reasonings were behind this strategy: 1) pre-incubation could allow the reporter protein to form a complex with PEX5 with no competition from the PTS1 proteins present in the PNS, thus providing a kinetic advantage to the reporter protein in the subsequent import reaction; and, 2) since in our *in vitro* assays small

amounts of the reticulocyte lysate containing the reporter protein are used, the amount of recombinant PEX5 that would be carried over into the import reactions would also be small, avoiding the competition problem at the DTM referred to above. Indeed, the use of this strategy resulted in a remarkable improvement of the *in vitro* import yields of some PTS1 proteins.

One of these proteins is Sterol Carrier Protein x (SCPx), a peroxisomal matrix protein that contains a canonical PTS1 and participates in the last step of the peroxisomal β-oxidation of fatty acids (review in (Gallegos *et al*, 2001)). SCPx is the only peroxisomal thiolase of the β-oxidation pathway capable of catalyzing the thiolytic cleavage of branched-chain fatty acids (Wanders *et al*, 1997; Atshaves *et al*, 2007). Its importance in human health is underscored by the existence of a SCPx deficiency that leads to dystonia, motor neuropathy and advanced leukoencephalopathy (Ferdinandusse *et al*, 2006). SCPx is a 59-kDa protein, homodimeric in its native state, which can be structurally divided into two domains: an amino-terminal thiolase and a carboxy-terminal sterol carrier protein-2 (SCP2) (Antonenkov *et al*, 2000; Seedorf *et al*, 1994). *In vivo*, SCPx is partially cleaved in the peroxisomal matrix, originating two proteins with different activities: a 46-kDa enzymatically active branched-chain fatty acid thiolase, and a 13-kDa SCP2 known to bind phospholipids, fatty acids, and fatty-acyl-CoA with high affinity and supposed to participate in lipid traffic, signaling, and metabolism (Ossendorp *et al*, 1996; Wirtz, 1997; Gallegos *et al*, 2001; Antonenkov *et al*, 1997; Otera *et al*, 2001; Seedorf *et al*, 1994; Schroeder *et al*, 2007). Recently, the peroxisomal processing protease TYSND1 was identified as the peptidase cleaving SCPx (as well as ACOX1 and PTS2 proteins) (Kurochkin *et al*, 2007; Mizuno *et al*, 2013).

As shown in Figure 6, when radiolabeled SCPx was pre-incubated with recombinant PEX5 before the *in vitro* import reaction, the amounts of protease-protected ^{35}S-SCPx obtained in the import assay were dramatically improved (compare lanes 3 and 4). It is important to note that the two import reactions shown in Figure 6 are chemically identical and differ solely in the step of the protocol where

recombinant PEX5 was added. The huge increase in the *in vitro* import yield of ^{35}S-SCPx obtained using this strategy is probably due to the formation of a complex between PEX5 and SCPx, as explained above. Indeed, upon sucrose gradient centrifugation (Figure 7), it is possible to observe that when ^{35}S-SCPx is pre-incubated with PEX5, the sedimentation coefficient of ^{35}S-SCPx changes: in the absence of PEX5, ^{35}S-SCPx sediments mainly in fraction 4, above bovine serum albumin (BSA) (fraction 5), whereas in the presence of PEX5, ^{35}S-SCPx peaks instead below BSA, in fractions 5-6.

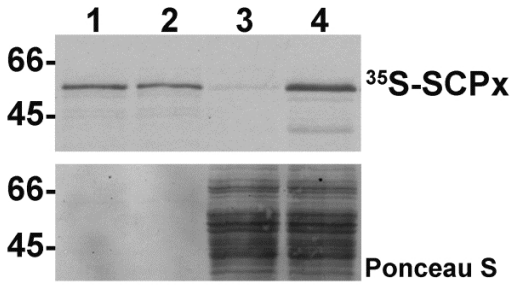


Figure 6. *In vitro* import efficiency of ^{35}S-SCPx can be dramatically improved by pre-incubation with recombinant PEX5.

Two chemically identical import reactions were assembled, differing solely in the step of the protocol where recombinant PEX5 was added. In one reaction (lane 3), ^{35}S-SCPx was pre-incubated in the absence of PEX5, and added to a PNS in ATP-containing import buffer. After addition of recombinant PEX5, the reaction was incubated for 15 min at 37 ºC. In the other reaction (lane 4), ^{35}S-SCPx was pre-incubated with recombinant PEX5, added to a PNS in the same buffer and incubated under the same conditions. Proteinase K-treated organelles were then subjected to SDS-PAGE/Western-blotting/autoradiography. Lanes 1 and 2 contain 5% of the pre-incubated ^{35}S-SCPx proteins used in the assays shown in lanes 3 and 4, respectively. The autoradiograph (upper panel) and the Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of protein standards in kDa.

It is important to note that when radiolabeled SCPx is incubated in the absence or presence of recombinant PEX5 and subjected to PK digestion, no radiolabeled SCPx can be detected (Figure 8A), indicating that ^{35}S-SCPx is highly susceptible to PK, a property that is not altered by the presence of recombinant PEX5. Similarly, and in contrast to the endogenous protein, when radiolabeled SCPx is simply mixed with PNS on ice, and treated with increasing amounts of PK, no protease-resistant ^{35}S-SCPx can be observed (Figure 8B), suggesting that its

susceptibility to PK is not affected by the presence of organelles. Furthermore, in the presence of a mild detergent such as TX-100, both radiolabeled SCPx and endogenous SCPx are degraded by PK, suggesting that the two proteins are intrinsically susceptible to the protease.

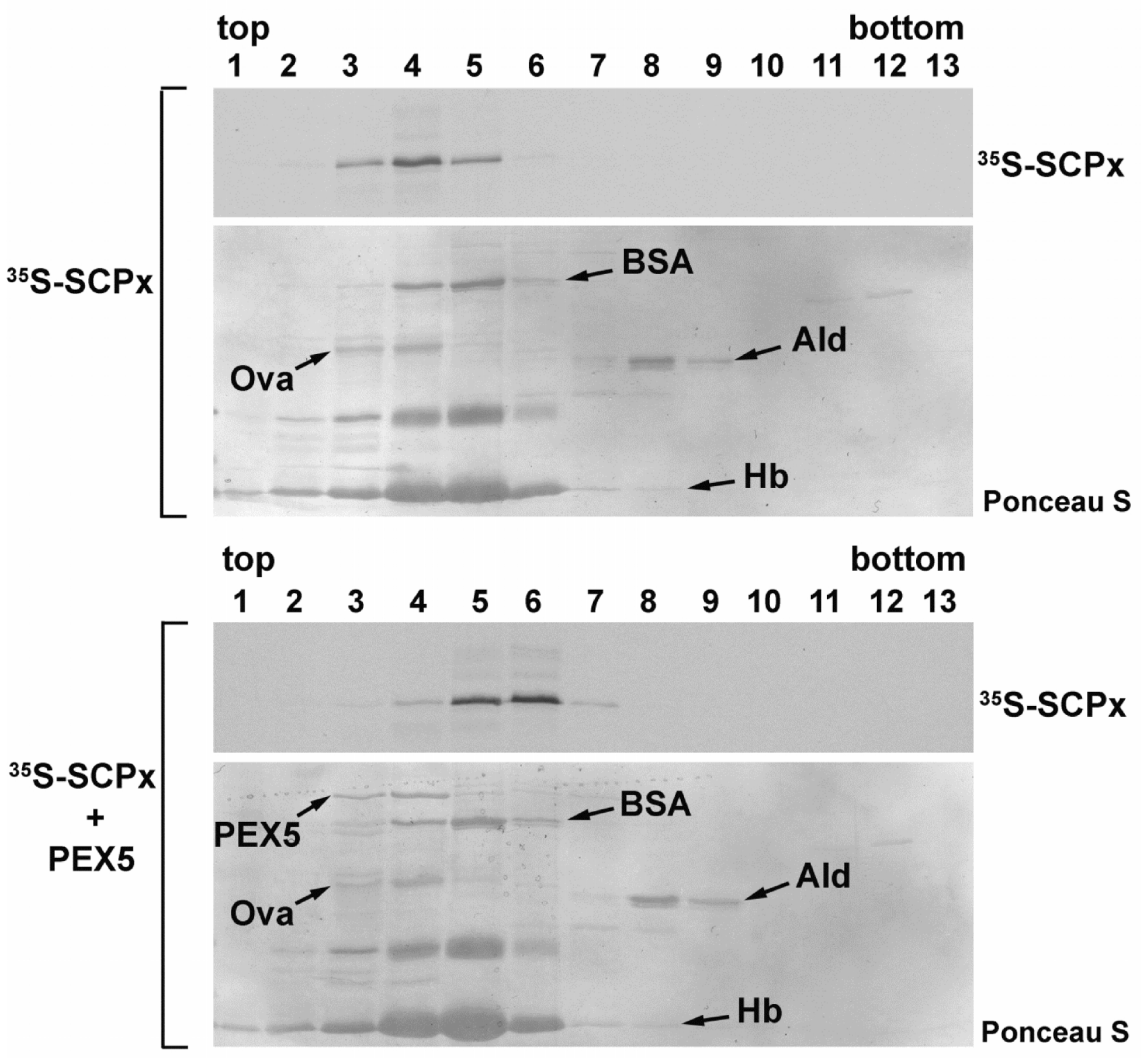


Figure 7. *In vitro* synthesized ^{35}S-SCPx interacts with PEX5.
^{35}S-SCPx was pre-incubated for 30 min at room temperature in the absence (upper panels) or presence (lower panels) of 1 µM recombinant PEX5. After adding a mixture of protein standards, the samples were subjected to sucrose gradient centrifugation. After fractionation, equivalent aliquots were subjected to SDS-PAGE/Western-blotting. Note that the sedimentation coefficient of ^{35}S-SCPx increases in the presence of PEX5, indicating that the two proteins interact. Autoradiographs and the Ponceau S-stained membranes are shown. Protein standards used were: ovalbumin (Ova; 45 kDa), bovine serum albumin (BSA; 66 kDa) and aldolase (Ald; 140 kDa).

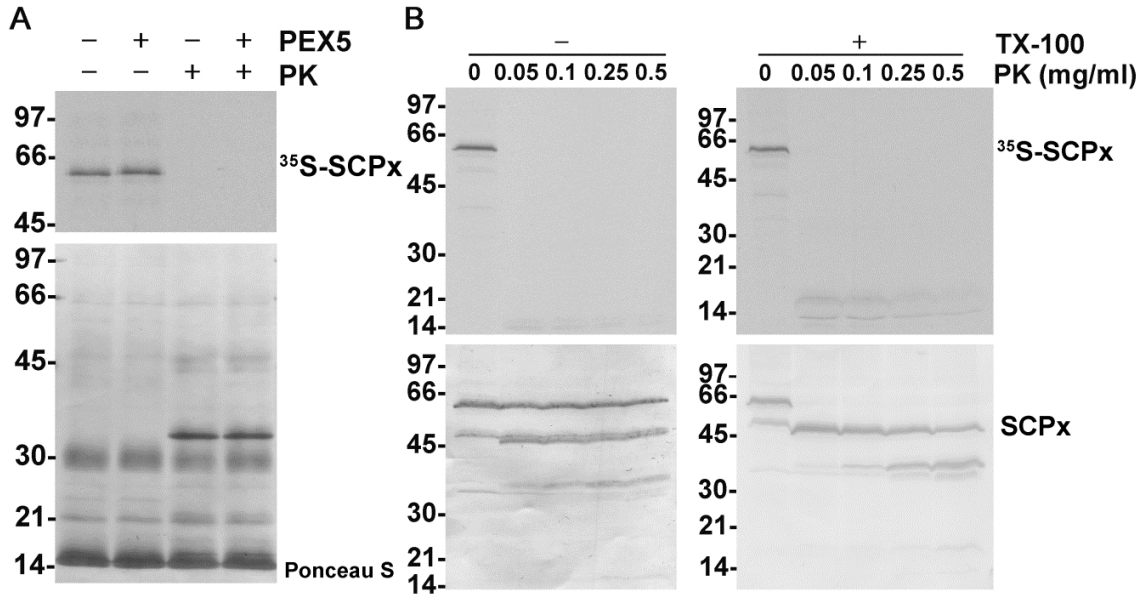


Figure 8. ^{35}S-SCPx is highly susceptible to proteinase K (PK).
A) ^{35}S-SCPx incubated in the absence or presence of recombinant PEX5 was treated with PK as indicated. After inactivation of the protease, the samples were subjected to TCA precipitation, and analyzed by SDS-PAGE/Western-blotting. An autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. **B)** Five microliters of ^{35}S-SCPx were mixed with 2 mg of PNS in import buffer containing or not 1% (w/v) TX-100, as indicated. Aliquots containing 400 µg of PNS were then treated with the indicated amounts of PK and processed as above. The membranes were exposed to an x-ray film (upper panels) and afterwards probed with an anti-SCPx antibody to detect endogenous SCPx (lower panels). Numbers to the left indicate the molecular masses of protein standards in kDa.

If the acquisition of a protease-protected status by SCPx in import assays really reflects its import into peroxisomes, then protease treatment in the presence of detergents of organelles containing imported ^{35}S-SCPx, should result in the proteolysis of the radiolabeled protein. Indeed, as shown in Figure 9, the protease-resistant status of imported ^{35}S-SCPx vanishes in the presence of a detergent (upper panel). This result thus suggests that the protease-resistant status of imported SCPx derives from a protection effect exerted by a lipid membrane.

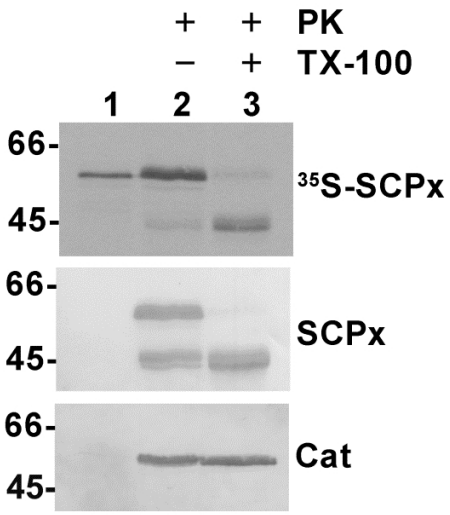


Figure 9. Imported ^{35}S-SCPx is protected from Proteinase K (PK) degradation by a lipid membrane.

Organelles from an *in vitro* import reaction were resuspended in import buffer and treated with PK in the absence (lane 2) or presence of TX-100 (lane 3). After protease inactivation and TCA precipitation, samples were analyzed by SDS-PAGE and Western-blotting/autoradiograhy. The behaviors of endogenous SCPx and catalase (Cat) are shown. Please note that the behavior of endogenous catalase, used here as a control, is different, because mouse liver catalase is quite resistant to this protease. Lane 1 contains 5% of the radiolabeled SCPx used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

4.1.1. Validation of the PTS1-centered *in vitro* import system developed

Several experiments were performed to characterize the properties and the specificity of this *in vitro* import system as well as to confirm that the organelle targeted by ^{35}S-SCPx is indeed the peroxisome.

We first looked at basic aspects of SCPx import, namely its time- and temperature-dependence. As shown is Figure 10A, the amount of organelle-associated protease-resistant ^{35}S-SCPx increases over time. *In vitro* import of radiolabeled SCPx is also temperature-dependent (see Figure 10B). At low temperatures the import efficiencies are significantly lower than those obtained at 26 ºC or 37 ºC. This result is in perfect agreement with the previous data obtained for PEX5 ((Costa-Rodrigues *et al*, 2004); see also section 4.3.).

If the acquisition of an organelle-associated protease-protected status of ^{35}S-SCPx is indeed related to the PEX5-mediated import process of SCPx, then replacing PEX5 by PEX5(N526K), a PEX5 mutant protein that is unable to efficiently bind PTS1 proteins (Dodt *et al*, 1995; Gatto & Geisbrecht, 2000; Carvalho *et al*, 2007a), should yield a negative result in our import assays. Indeed, as shown in Figure 11, this was exactly the result obtained (Figure 11, compare lanes 3 and 4). A similar negative result was obtained when ^{35}S-SCPx was pre-incubated with both recombinant PEX5 and a molar excess of a protein comprising the PTS1-binding domain of PEX5 (TPRs; Figure 11, lane 5). The latter protein can still bind PTS1 proteins efficiently but lacks the N-terminal domain of PEX5 required for a productive interaction with the peroxisomal DTM (Szilard & Rachubinski, 2000). The inhibitory effect of TPRs requires its PTS1-binding activity because a mutant version of this protein carrying the N526K mutation [TPRs(N526K)] does not interfere with the PEX5-mediated import of radiolabeled SCPx (Figure 11, lane 6). When ^{35}S-SCPx was pre-incubated with PEX5 plus a recombinant protein comprising the N-terminal domain of PEX14 (NDPEX14), a component of the DTM, no protease-resistant ^{35}S-SCPx was detected (Figure 11, lane 7). As explained in detail in section 1.3.3.1., this domain of PEX14 binds with high affinity to the so-called diaromatic motifs present in the N-terminal half of PEX5 (Schliebs *et al*, 1999) which are essential for the PEX5 interaction with the DTM (Otera *et al*, 2002). NDPEX14 may also trigger the release of the cargo protein, as recently proposed (Freitas *et al*, 2011). No such effect was obtained when another recombinant peroxin (PEX19) was used in these experiments (in Figure 11, compare lanes 7 and 9, respectively). PEX19, as mentioned in section 1.3.1., is involved in a different pathway of peroxisome biogenesis and was used here simply as a negative control. Altogether, these *in vitro* assays show that import of radiolabeled SCPx is dependent on an available and functional full-length PEX5.

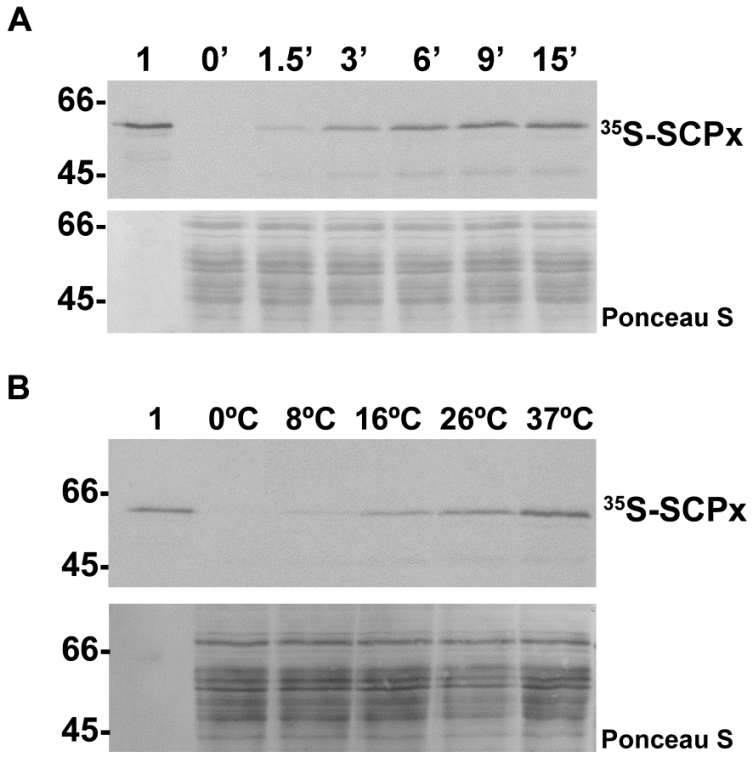


Figure 10. The *in vitro* import of ^{35}S-SCPx is time- and temperature-dependent.
A) ^{35}S-SCPx pre-incubated with recombinant PEX5 was subjected to a standard import assay. Aliquots of the reaction were withdrawn at the indicated time points and treated with PK. After protease inactivation, organelles were isolated by centrifugation and processed for SDS-PAGE/autoradiography (upper panel). The Ponceau S-stained membrane is also shown (lower panel). **B)** Radiolabeled SCPx pre-incubated with recombinant PEX5 was subjected to import assays in the presence of ATP for 15 min at the indicated temperatures. Samples were processed as described in A). Lane 1 in A) and B) contains 5% of the radiolabeled SCPx used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

To confirm that the membrane-bound organelle to which PEX5 targets ^{35}S-SCPx is in fact the peroxisome, a protease-treated import reaction was loaded onto a discontinuous Nycodenz gradient and centrifuged. The gradient was subsequently fractionated and equal aliquots of each fraction were subjected to SDS-PAGE/Western-blotting and autoradiography. This technique explores the fact that peroxisomes display a density higher than other organelles present in mouse liver PNS (Hartl *et al*, 1985). As shown in Figure 12, most mitochondria and microsomes are found in fractions 5-10 and 8-11, respectively, as assessed using organelle-specific antibodies (anti-cytochrome c and anti-KDEL, respectively). Peroxisome

gradient distribution was assessed using antibodies directed to catalase and endogenous SCPx. In contrast to mitochondria and microsomes, most peroxisomes are found in fractions 1-3, near the bottom of the gradient, and represent highly pure intact peroxisomes (Gouveia *et al*, 2003a; Pinto *et al*, 2006). We note that a major fraction of catalase is also detected at the top of the gradient (fractions 10-13). This population represents mostly catalase that has leaked from peroxisomes during preparation of the PNS (Gouveia *et al*, 2003a; Pinto *et al*, 2006). Unlike non-peroxisomal/soluble SCPx, which is proteinase K-sensitive, non-peroxisomal mouse liver catalase is quite resistant to this protease (see Figure 6, lower panel). Importantly, the distribution of *in vitro* imported radiolabeled SCPx parallels the one of the endogenous mouse liver SCPx, *i.e.*, it is found essentially in fractions 1-3, where highly pure peroxisomes sediment. Collectively, these data show that ^{35}S-SCPx is efficiently imported into peroxisomes *in vitro*.

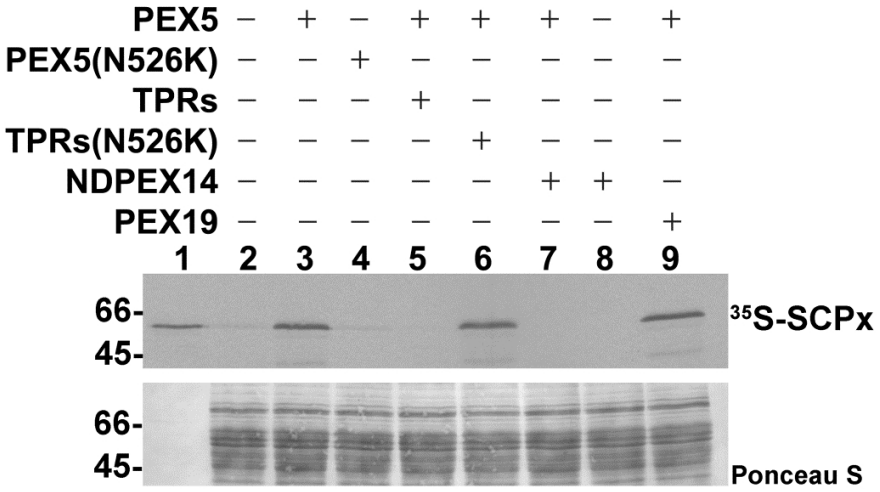


Figure 11. ^{35}S-SCPx *in vitro* import depends on the peroxisomal receptor PEX5.
^{35}S-SCPx was pre-incubated in the absence (-) or presence (+) of the indicated recombinant proteins. Protein mixtures were then subjected to standard import assays and analyzed as described in Figure 10. Lane 1 contains 5% of the radiolabeled SCPx used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

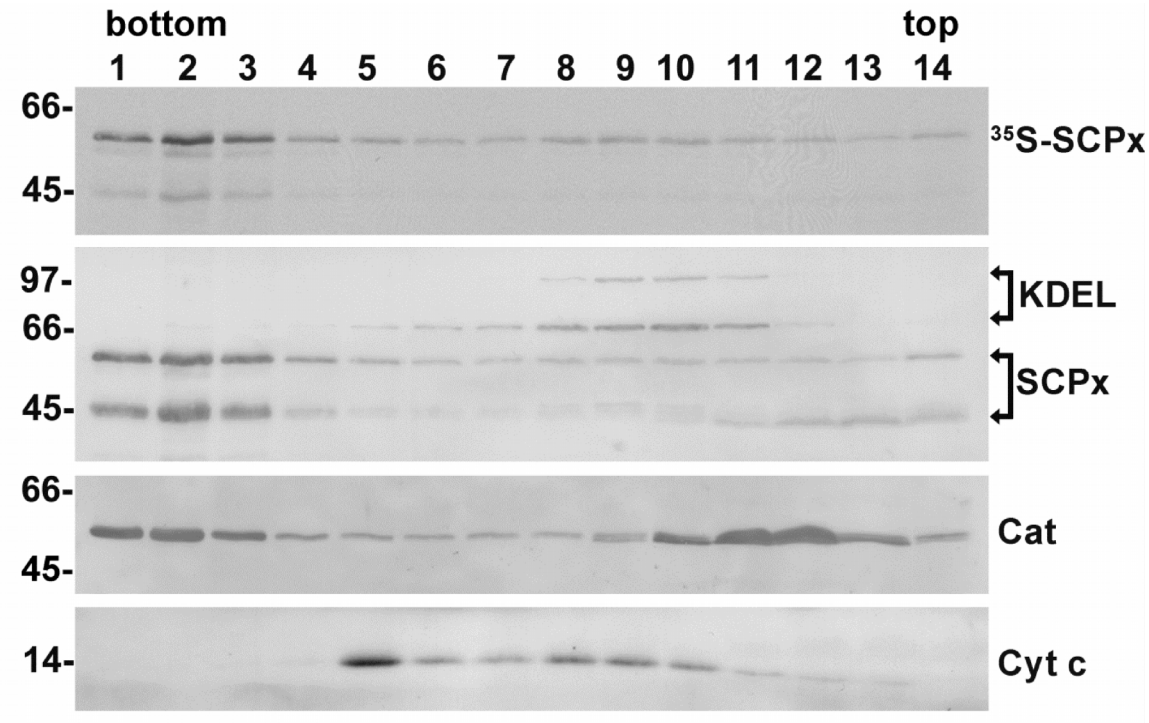


Figure 12. Imported ^{35}S-SCPx co-localizes with peroxisomes in a Nycodenz gradient.
A protease-treated import reaction was subjected to Nycodenz gradient centrifugation. The behaviors of ^{35}S-SCPx, endogenous SCPx, catalase (Cat), cytochrome c (Cyt c; a mitochondrial marker) and two endoplasmic reticulum proteins (KDEL; recognizes GRP72 and GRP98) are shown. The fraction of catalase detected at the top of the gradient represents mostly catalase that has leaked from peroxisomes during PNS preparation. Unlike soluble SCPx, soluble mouse catalase is quite resistant to proteinase K. Numbers to the left indicate the molecular masses of protein standards in kDa.

Having established the robustness and specificity of this PTS1-centered *in vitro* import system, the next aim of this work was to characterize the translocation mechanism of a PTS1 protein across the peroxisomal membrane.

4.2. Characterization of the translocation mechanism of a PTS1 cargo protein across the peroxisomal membrane

4.2.1. ^{35}S-SCPx import into peroxisomes does not require cytosolic ATP hydrolysis or PEX5 monoubiquitination

We know from previous work (Oliveira *et al*, 2003) that most of the DTMs in a PNS fraction are occupied by endogenous PEX5. In the absence of ATP, this PEX5

population will not be extracted back into the cytosol by the REM and thus the import yields obtained in these conditions are very low and cannot be compared with those observed in the presence of ATP. To circumvent this problem, the PNS used in all experiments described below was subjected to a priming step consisting of a 5 min incubation in the presence of 0.3 mM ATP. The priming step thus ensures that regardless of the energetic conditions used in the subsequent import assays, the number of free DTMs is the same at time 0 of the different assays. Naturally, this number will decrease over time if the assays are made in the presence of AMP-PNP or apyrase (see below). However, this effect can be minimized by performing import assays for a short period of time (*i.e.*, 7 min) (Alencastre *et al*, 2009).

Taking into account these properties of our *in vitro* import system, we then asked whether or not import of SCPx requires hydrolysis of cytosolic ATP. Two different strategies were used for this purpose. In the first, a PNS that had been primed for import was incubated with a vast excess (3 mM) of AMP-PNP, before adding the radiolabeled reporter protein. AMP-PNP is a potent inhibitor of ATPases that cleave the bond between the β- and γ-phosphate groups of ATP, *i.e*., that hydrolyze ATP to ADP. It should be noted that ubiquitination of PEX5 at the DTM still occurs in the presence of AMP-PNP because the ubiquitin-activating enzyme uses this ATP analog quite efficiently (Grou *et al*, 2012; Haas *et al*, 1983). However, the export of monoubiquitinated PEX5 from the DTM to the cytosol, a process catalyzed by the ATPases PEX1/PEX6, is completely blocked by AMP-PNP (Grou *et al*, 2012). As shown in Figure 13, the import efficiencies of radiolabeled SCPx in reactions supplemented with either 3 mM ATP (lane 4) or 3 mM AMP-PNP (lane 5) are essentially the same. Thus, a 10-fold molar excess of AMP-PNP over ATP does not result in an inhibition of SCPx import although export of monoubiquitinated PEX5 is blocked under the same conditions (see Figure 14A, compare lanes 3 and 5), as expected (Grou *et al*, 2012).

In the second strategy both ^{35}S-SCPx and the primed PNS were treated with apyrase, an enzyme that hydrolyzes ATP and other NTPs (Hwang & Schatz, 1989), before the import reaction. This apyrase treatment did not affect the import efficiency of radiolabeled SCPx (Figure 13, compare lanes 4 and 6). An *in vitro* import control

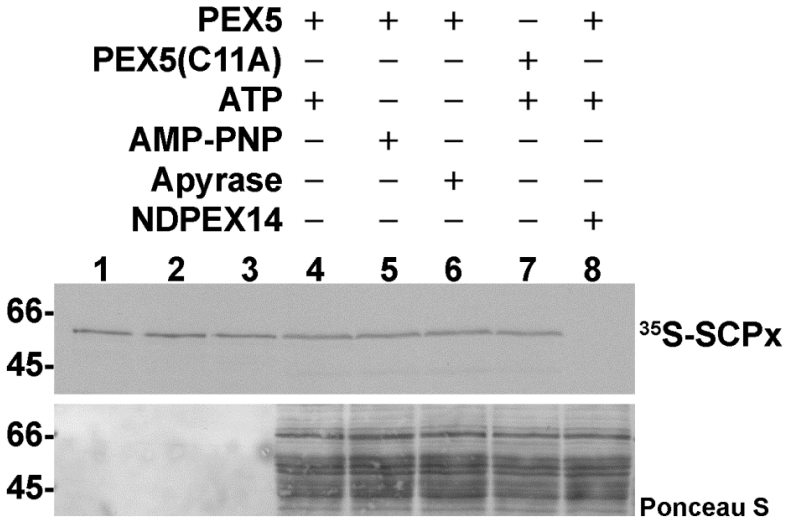


Figure 13. *In vitro* import of ^{35}S-SCPx does not require cytosolic ATP hydrolysis or ubiquitination of PEX5.

^{35}S-SCPx was pre-incubated either in the presence of recombinant PEX5 (lanes 1 and 2) or PEX5(C11A) (lane 3). An aliquot of the PEX5-containing ^{35}S-SCPx was further treated with apyrase (lane 2). These samples were then subjected to import assays, as follows: lane 4 – assay containing ATP and ^{35}S-SCPx pre-incubated with PEX5; lane 5 – the same as in lane 4 but in the presence of AMP-PNP instead of ATP; lane 6 – assay containing ^{35}S-SCPx pre-incubated with PEX5 and PNS, both pre-treated with apyrase; lane 7 – import assay containing ATP and ^{35}S-SCPx pre-incubated with PEX5(C11A); lane 8 – the same as in lane 4 but also containing recombinant NDPEX14. Samples were processed as described in Figure 10. Lanes 1, 2 and 3 contain 5% of the ^{35}S-SCPx samples used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

experiment using ^{35}S-PEX5 as the reporter protein, shows that the apyrase treatment efficiently depletes ATP from the reactions because no ubiquitination of PEX5 is observed under these conditions (Figure 14B, compare lanes 3 and 4), as described before (Alencastre *et al*, 2009). Altogether, these results strongly suggest that import of radiolabeled SCPx into peroxisomes does not require monoubiquitination of PEX5 or the hydrolysis of cytosolic ATP. Additional data supporting the first of these conclusions was obtained when radiolabeled SCPx was pre-incubated with a recombinant mutated version of PEX5, PEX5(C11A). PEX5(C11A) possesses an alanine at position 11 instead of the conserved cysteine that is required for monoubiquitination and subsequent export of PEX5. As alanine residues cannot be ubiquitinated, PEX5(C11A) is an import-competent, but export-incompetent PEX5 protein (Grou *et al*, 2009b). As shown in Figure 13, albeit non-ubiquitinable,

PEX5(C11A) is as efficient as PEX5 in promoting the import of the cargo (compare lanes 4 and 7).

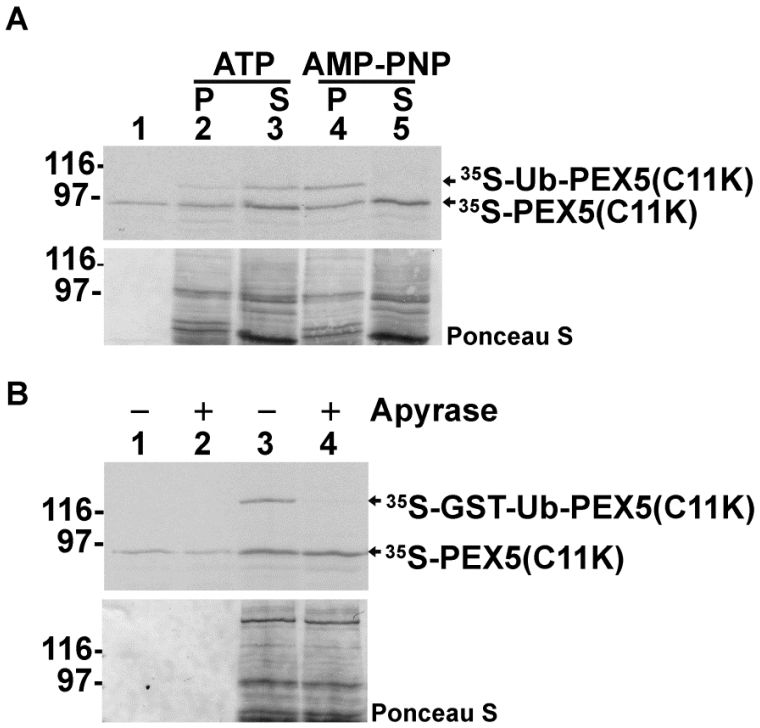


Figure 14. Control experiments showing the effect of AMP-PNP and apyrase on PEX5 export and monoubiquitination, respectively.

A) Export of monoubiquitinated ^{35}S-PEX5(C11K) (^{35}S-Ub-PEX5(C11K)) is completely blocked by AMP-PNP. ^{35}S-labeled PEX5(C11K) was subjected to *in vitro* import reactions containing ubiquitin aldehyde (Ubal; an inhibitor of deubiquitinases) and either ATP (lanes 2 and 3) or AMP-PNP (lanes 4 and 5). After 7 min at 37 ºC, the import reactions were centrifuged to obtain an organelle pellet (P) and a supernantant (S) and both fractions were analyzed by SDS-PAGE/Western-blotting. Note that AMP-PNP allows PEX5(C11K) ubiquitination but not its export into the soluble phase of the import reaction (Grou *et al*, 2012). Lane 1 contains 5% of the ^{35}S-labeled PEX5(C11K) used in the assays.

B) Monoubiquitination of ^{35}S-PEX5(C11K) is completely blocked by the apyrase treatment. ^{35}S-labeled PEX5(C11K) was incubated in the absence (-) or presence (+) of apyrase (lanes 1 and 2, respectively). The first of these samples (minus apyrase) was subjected to an *in vitro* import assay in the presence of ATP (lane 3); the apyrase-treated ^{35}S-labeled PEX5(C11K) was subjected to an import assay using apyrase-treated PNS (lane 4). Both reactions contained also 15 µM of GST-Ub. The organelles were then isolated by centrifugation and analyzed by SDS-PAGE/Western-blotting/autoradiography. Note that GST-Ub is efficiently used by the machinery that monoubiquitinates PEX5 but, in contrast to ubiquitin, results in a PEX5 species that is not exported from the DTM (Carvalho *et al*, 2007b; Grou *et al*, 2009b). Also, monoubiquitinated PEX5(C11K) is more stable than monoubiquitinated PEX5 upon SDS-PAGE. This property increases the sensitivity of the ubiquitination assays (Grou *et al*, 2009b). Lanes 1 and 2 contain 5% of the ^{35}S-labeled PEX5(C11K) used in the assays.

4.2.2. ^{35}S-DECR2 is also imported into peroxisomes in a PEX5 monoubiquitination- and cytosolic ATP-independent manner

The results above strongly suggest that SCPx can be specifically imported to peroxisomes without requiring cytosolic ATP consumption or PEX5 monoubiquitination. To assess whether this property of SCPx import can be extended to other PTS1 proteins, we repeated the experiments described in the previous section (see section 4.2.1.) but this time using ^{35}S-2,4-Dienoyl-CoA Reductase (^{35}S-DECR2).

DECR2 is an auxiliary enzyme of the peroxisomal β-oxidation pathway, necessary for the complete degradation of mono or poly-unsaturated fatty acids with double bonds (Fransen *et al*, 1999). This 33-kDa protein is possibly tetrameric in its native state and contains a canonical PTS1 signal (AKL) (Fransen *et al*, 1999; Hua *et al*, 2012).

As shown in Figure 15, the import efficiencies of radiolabeled DECR2 are essentially the same in reactions supplemented with either ATP (lane 4) or AMP-PNP (lane 5). Similarly, when ^{35}S-DECR2 and the primed PNS were treated with apyrase before the import reaction, the import efficiency of radiolabeled DECR2 was also not decreased (Figure 15, compare lanes 4 and 6), suggesting that PEX5 monoubiquitination is also not a requirement for the import of this PTS1 protein. This conclusion was further corroborated in an assay where recombinant PEX5 was replaced by PEX5(C11A). No differences were detected when comparing the efficiencies of PEX5 or PEX5(C11A) in the import of DECR2 (Figure 15, compare lanes 4 and 7).

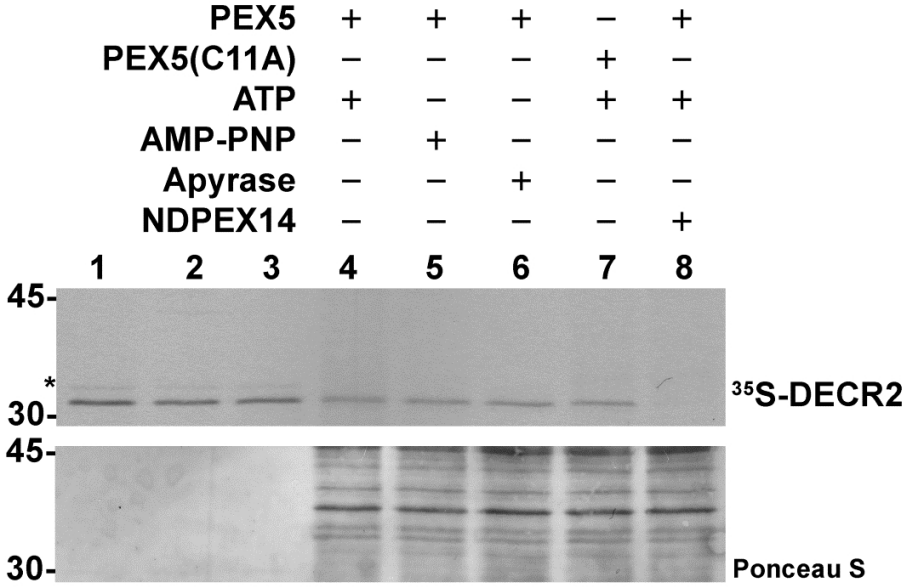


Figure 15. *In vitro* import of ^{35}S-DECR2 does not require cytosolic ATP hydrolysis.
^{35}S-labeled 2,4-dienoyl-CoA reductase (^{35}S-DECR2) was pre-incubated either in the presence of recombinant PEX5 (lanes 1 and 2) or PEX5(C11A) (lane 3). An aliquot of the PEX5-containing ^{35}S-DECR2 was further treated with apyrase (lane 2). These samples were then subjected to import assays, as follows: lane 4 – assay containing ATP and ^{35}S-DECR2 pre-incubated with PEX5; lane 5 – the same as in lane 4 but in the presence of AMP-PNP instead of ATP; lane 6 – assay containing ^{35}S-DECR2 pre-incubated with PEX5 and PNS, both pre-treated with apyrase; lane 7 – import assay containing ATP and ^{35}S-DECR2 pre-incubated with PEX5(C11A); lane 8 – the same as in lane 4 but also containing recombinant NDPEX14. Samples were processed as described in Figure 10. Lanes 1, 2 and 3 contain 5% of the ^{35}S-DECR2 samples used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

4.2.3. ^{35}S-SCPx is released into the peroxisomal matrix before PEX5 monoubiquitination

The experiments presented above, indicate that ^{35}S-SCPx acquires a protease-protected peroxisomal location in a process that requires PEX5, but not its ubiquitination, nor hydrolysis of cytosolic ATP. However, it remains unclear whether the protease-protected ^{35}S-SCPx detected in all those experiments represents a protein that was already translocated into the peroxisomal matrix or a species that is still associated with the DTM. To clarify this issue, radiolabeled SCPx was pre-incubated with either PEX5 or PEX5(C11A) and subjected to import assays in the presence of ATP or AMP-PNP or apyrase, as indicated in Figure 16. After protease

treatment, the organelles were isolated by centrifugation, disrupted by sonication and ultracentrifuged to obtain membrane (lanes P) and soluble (lanes S) fractions. The efficiency of the fractionation procedure was assessed by Western-blotting using antibodies directed to PEX14 (a peroxisomal intrinsic membrane protein), cytochrome c (a peripheral membrane mitochondrial protein) and catalase (a soluble peroxisomal matrix protein). The results obtained show that cytochrome c and PEX14 are mostly found in the membrane fraction, whereas catalase is almost completely recovered in the soluble fraction, as expected. Although a major fraction of endogenous SCPx was found in the soluble fraction, some protein was also recovered in the membrane pellet. The reason why SCPx and catalase present slightly different behaviors may be

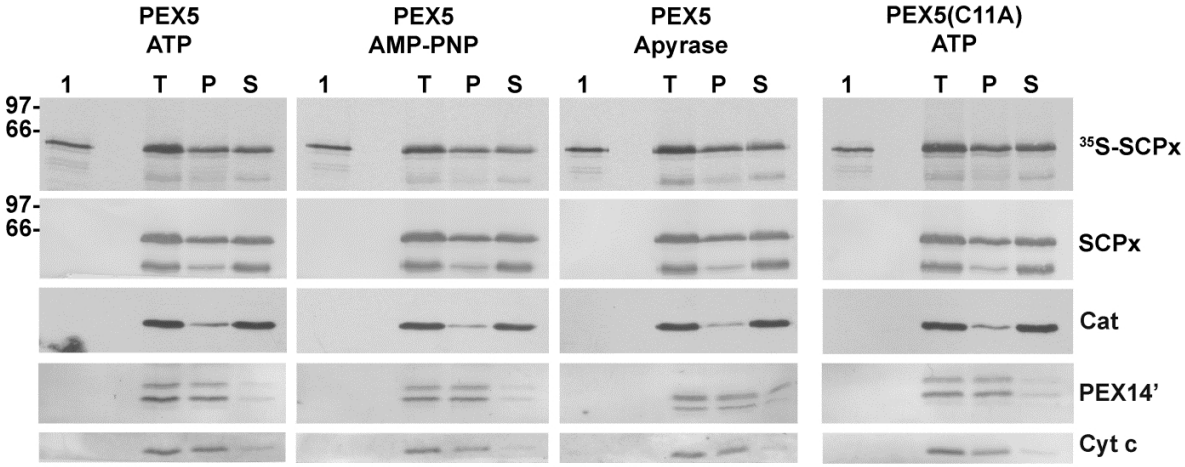


Figure 16. *In vitro* imported ^{35}S-SCPx behaves as endogenous SCPx upon fractionation of peroxisomes.

^{35}S-SCPx pre-incubated with the indicated PEX5 proteins was subjected to *in vitro* import assays under different energetic conditions, as specified. At the end of the import reaction, the organelles were treated with PK, isolated by centrifugation, and sonicated. One half of these samples was kept on ice (lanes T) whereas the other half was ultracentrifuged to obtain membrane (P) and soluble (S) fractions. Samples were analyzed by SDS-PAGE/Western-blotting/autoradiography. The behaviors of endogenous SCPx, catalase (Cat), PEX14, and cytochrome c (Cyt c) are also shown. Note that PEX14 is converted into small 16-18 kDa fragments (PEX14') upon PK treatment (see also (Oliveira *et al*, 2002)). Numbers to the left indicate the molecular masses of protein standards in kDa.

related to the intrinsic properties of the C-terminal half of SCPx. Indeed, it was demonstrated that the C-terminal SCP2 domain of SCPx is capable of binding membrane lipids (Seedorf *et al*, 1994). Importantly, and regardless of the experimental conditions used in the import assays, *in vitro* imported radiolabeled

SCPx displays exactly the behavior of endogenous SCPx, thus suggesting that it represents a species that was already translocated into the matrix of the organelle.

4.3. Docking and translocation are different steps of the PEX5-mediated protein import pathway

The data presented thus far suggest that import and translocation of radiolabeled SCPx across the peroxisomal membrane occurs upstream of the first cytosolic ATP-dependent step, *i.e.*, before PEX5 monoubiquitination. According to current models, there are only two events occurring at the peroxisome before this first ATP-dependent step: 1) docking of the PEX5-cargo protein complex at the DTM and 2) insertion of PEX5 into this machinery. It should be noted, however, that while there is several experimental evidence supporting the concept that PEX5 becomes inserted into the DTM in a cargo-dependent but ATP-independent manner (Gouveia *et al*, 2003b, 2000; Miyata & Fujiki, 2005; Oliveira *et al*, 2003), data regarding the docking step itself are still very scarce. Actually, it is presently unknown whether such a step really exists mechanistically, or if docking and insertion of PEX5 into the DTM are simply the beginning and the end of a single step. To discriminate between these two possibilities, we explored the fact that insertion of PEX5 into the DTM is inhibited at low temperatures (Costa-Rodrigues *et al*, 2004) and asked whether docking of the PEX5-cargo protein complex can still occur under those conditions. For this purpose, radiolabeled SCPx was pre-incubated with a mixture of recombinant and radiolabeled PEX5(C11A) and subjected to import assays at different temperatures allowing us to monitor the behavior of both the cargo protein and the receptor simultaneously. After the import reaction, samples were halved, treated or not with PK, and analyzed by SDS-PAGE/Western-blotting/autoradiography.

As shown in Figure 17, PEX5(C11A) cannot be inserted into the DTM at low temperatures (as assessed by the acquisition of a protease-resistant status; (Gouveia *et al*, 2003a; Costa-Rodrigues *et al*, 2004)); insertion of PEX5(C11A) into the DTM can only be detected at temperatures above 8 ºC (Figure 17, compare lanes 2-3 with lanes 4-6). Importantly, the same temperature dependence profile is observed for the import of SCPx, suggesting that it only occurs when PEX5 gets inserted into the DTM.

Interestingly, when the protease treatment was omitted, considerable amounts of both ^{35}S-labeled PEX5(C11A) and SCPx were found in the organelle fractions, even in import reactions performed at low temperatures (Figure 17, lanes 7-8). To assess if these proteins are specifically adsorbed to peroxisomes, radiolabeled SCPx and PEX5(C11A) pre-incubated as described above, were subjected to import assays at 0 ºC or 37 ºC in the presence of either recombinant ΔC1PEX5 or the negative control PEX19. As shown in Figure 18, a considerable fraction of both radiolabeled PEX5(C11A) and SCPx was indeed specifically adsorbed to the peroxisome because assays containing recombinant ΔC1PEX5, a PEX5 protein that lacks the PTS1-binding domain but that is still competent in entering the DTM (Gouveia *et al*, 2003b; Grou *et al*, 2012), have smaller amounts of the radiolabeled proteins than those observed in the presence of PEX19 (Figure 18, compare lanes 2 and 4 with lanes 6 and 8, respectively).

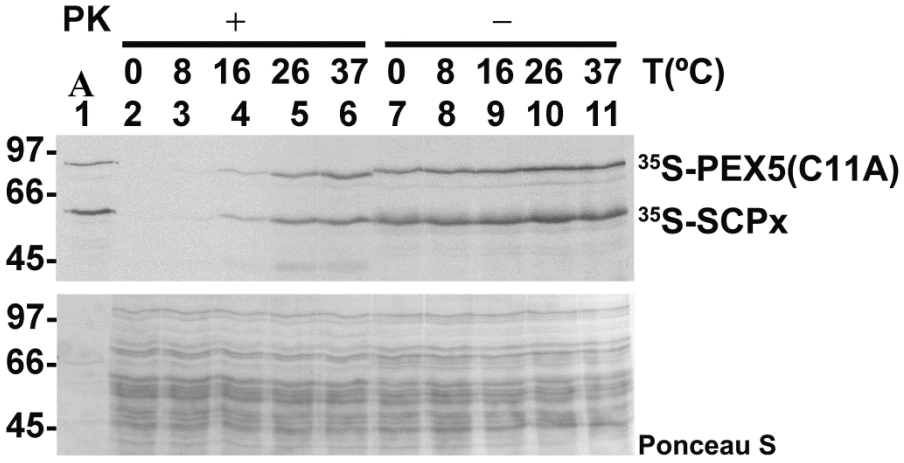


Figure 17. Temperature dependence of the docking/insertion of PEX5 into the DTM and SCPx import.

^{35}S-SCPx pre-incubated with a mixture of recombinant and ^{35}S-labeled PEX5(C11A) was subjected to import assays at different temperatures. After 15 min, the samples were halved and treated (lanes +) or not (lanes –) with proteinase K (PK). The organelles were analyzed by SDS-PAGE/autoradiography (upper panel). The Ponceau S-stained membrane is also shown (lower panel). Lane 1 contains 5% of the radiolabeled proteins used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

A similar competition phenomenon was observed when organelles isolated from an import assay performed at 0 ºC were washed in buffer containing ΔC1PEX5 but not when it contained PEX19 (Figure 19, compare lanes 3 and 4 with lanes 5 and 6, respectively). Thus, both ^{35}S-labeled PEX5(C11A) and SCPx can interact specifically and reversibly with peroxisomes.

Taken together, these data reveal the existence of a mechanistically distinct docking step of the PEX5-cargo protein complex at the DTM and suggest that import of SCPx occurs concomitantly with insertion of PEX5 into the DTM.

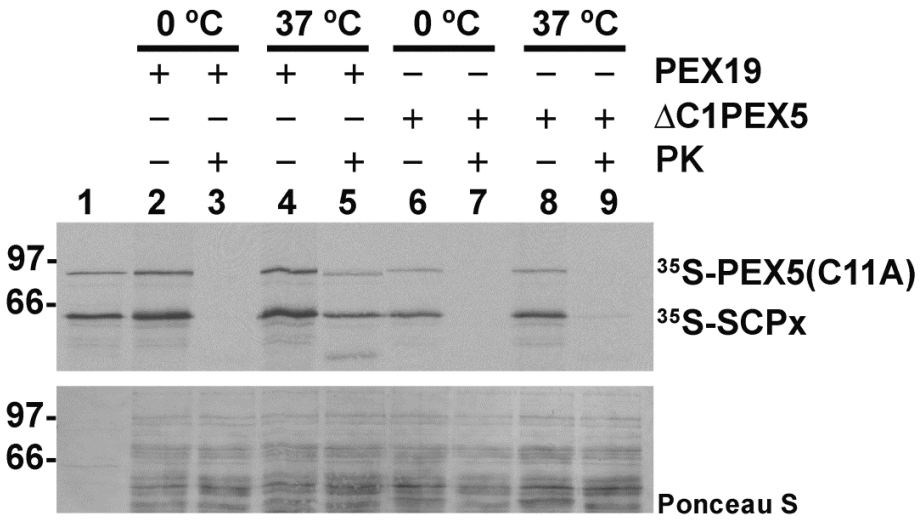


Figure 18. Specific docking of ^{35}S-PEX5(C11A) and ^{35}S-SCPx at the peroxisome.
^{35}S-SCPx pre-incubated with a mixture of recombinant and ^{35}S-labeled PEX5(C11A) was subjected to import assays at 0 ºC or 37 ºC in the presence of either recombinant ΔC1PEX5 or PEX19 (5 μM each), as indicated. Protease-treated and untreated organelles were then analyzed as in Figure 17. Lane 1 contains 5% of the radiolabeled proteins used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

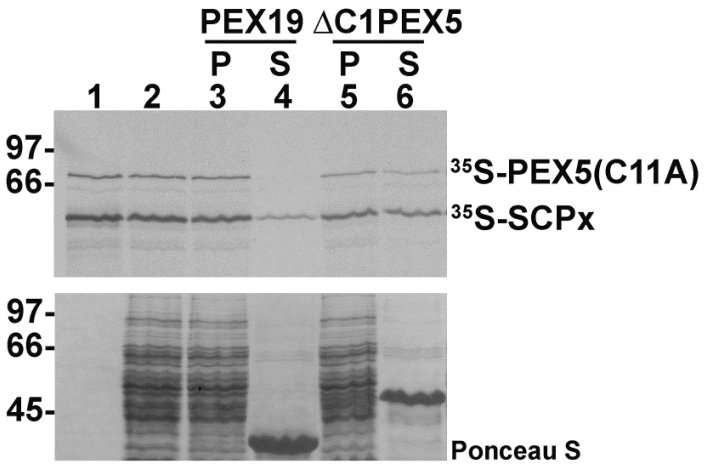


Figure 19. The docking step is reversible.
Organelles from an import assay containing ^{35}S-SCPx and ^{35}S-PEX5(C11A) performed at 0 ºC (lane 2) were resuspended in import buffer containing either recombinant ΔC1PEX5 or PEX19, incubated for 15 min and re-isolated by centrifugation. Organelle pellets (P) (lanes 3 and 5) and the corresponding supernatants (S) (lanes 4 and 6) were analyzed as in Figure 17. Lane 1 contains 5% of the radiolabeled proteins used in the assays. Numbers to the left indicate the molecular masses of protein standards in kDa.

5. DISCUSSION

In mammals and many other organisms, import of all cargo proteins into the peroxisomal matrix relies on the peroxisomal shuttling receptor, PEX5 (Otera *et al*, 1998; Braverman *et al*, 1998; Galland *et al*, 2007; Woodward & Bartel, 2005). The pathway followed by PEX5 during this process has been extensively studied over the years and is now known with reasonable detail (Grou *et al*, 2009a; Hu *et al*, 2012; Liu *et al*, 2012; Platta *et al*, 2012). However, crucial aspects of the import process such as the timing of the cargo translocation step are still poorly defined. To address these issues, a cargo-centered perspective was required. As referred in section 4.1., low import yields are usually obtained when using PTS1 proteins as reporters in *in vitro* import assays. Therefore, our laboratory has recently developed an *in vitro* import system centered in a PTS2 cargo protein, pre-thiolase (Alencastre *et al*, 2009). The data obtained strongly suggest that the translocation of pre-thiolase across the peroxisomal membrane as well as its processing and release into the peroxisomal matrix, occurs before PEX5 monoubiquitination and does not require cytosolic ATP hydrolysis (Alencastre *et al*, 2009). However, PTS2 proteins have the particularity to require PEX7 for their peroxisomal targeting, a protein that has been suggested to enter the organelle matrix together with the PTS2 proteins (Nair *et al*, 2004; Lazarow, 2006; Kunze *et al*, 2011; Braverman *et al*, 1997, 1998; Otera *et al*, 1998). Therefore, it remained unclear whether the mechanistic data gathered for PTS2 proteins is a general property of the peroxisomal protein import machinery or a unique feature of the PTS2 import pathway. The main aim of this work was thus to dissect the import mechanism of a PTS1 protein and thus to define the general properties of protein translocation across the mammalian peroxisomal membrane.

To fulfill this task, it was necessary to develop an efficient and robust PTS1-centered *in vitro* import system. To overcome the competition problems imposed by endogenous soluble PTS1 proteins, a new strategy was developed: the reporter PTS1 protein was pre-incubated with recombinant PEX5 prior to the *in vitro* import reaction. This pre-incubation step allows the reporter protein to interact with PEX5 with no competition from endogenous PTS1 proteins of the PNS, and resulted in a remarkable improvement of the *in vitro* import yields of several PTS1 proteins, including SCPx (this work and unpublished results).

After establishing the robustness and specificity of this PTS1-centered *in vitro* import system, the import mechanism of SCPx was characterized. The results obtained strongly suggest that the import of radiolabeled SCPx into peroxisomes does not require monoubiquitination of PEX5 or the hydrolysis of cytosolic ATP. To exclude the possibility that the ability to be imported into peroxisomes in a PEX5 monoubiquitination- and cytosolic ATP-independent manner is a particularity of SCPx, identical experiments were performed with another PTS1 protein, DECR2. Similarly, neither the use of the non-hydrolyzable ATP analog, AMP-PNP, nor the depletion of ATP from import reactions affected the PEX5-mediated import of DECR2.

The fact that a protein acquires a protease-protected status when subjected to an import assay suggests that no major portion of its polypeptide chain is exposed into the cytosol, but does not discriminate between a protein that has already translocated into the peroxisomal matrix from a species that is still associated with the DTM. To clarify this issue, protease-treated organelles were disrupted by sonication, and separated into soluble and membrane fractions. Remarkably and independently of the energetic conditions used, the behavior of radiolabeled SCPx in these experiments was exactly the one observed for endogenous SCPx, thus suggesting that the radiolabeled protein had already translocated into the matrix of the organelle. Consequently, neither ubiquitination of PEX5 at the DTM, nor the ATP-dependent extraction of monoubiquitinated PEX5 from the DTM, seem to play a role in the release of the cargo protein from the DTM into the peroxisomal matrix.

The conclusion that import of a PTS1 protein occurs upstream of PEX5 monoubiquitination immediately indicates that translocation of the cargo protein across the organelle membrane occurs during the docking/insertion of PEX5 at/into the DTM. By performing import reactions at several temperatures, it was possible to determine for the first time that the docking at the peroxisomal membrane and insertion of PEX5 into the DTM are two independent steps. Importantly, the temperature-dependence profiles of both radiolabeled PEX5 insertion into the DTM and radiolabeled SCPx import were the same, strongly supporting the idea that the two events are coupled.

Presently, there are two major models aiming at explaining how peroxisomal matrix proteins reach the organelle matrix. The first model was proposed by our laboratory several years ago and postulates that the driving force for protein translocation across the organelle membrane resides in the strong protein-protein interactions established between PEX5 and the DTM (Oliveira *et al*, 2003; Azevedo *et al*, 2004). According to this model, chemical energy input (*i.e.*, ATP hydrolysis) is only necessary to extract PEX5 from the DTM, that is, to reset the peroxisomal import machinery. The data supporting this model are abundant and have been summarized previously (see section 1.3.3.2.).

The second model was recently published by the Erdmann group under the name “export-driven protein import model” (Schliebs *et al*, 2010). For the sake of completeness, we must note that this “model”, as proposed by the authors, actually comprises two different models. The first version proposes that ubiquitination/export of the shuttling receptors is necessary to generate free DTM so that protein import can continue. In other words, ATP is needed to reset the PIM. Obviously, this version of the “export-driven protein import model” is nothing else other than the model our laboratory proposed several years before (Oliveira *et al*, 2003; Azevedo *et al*, 2004) and has been refining since then (Grou *et al*, 2009a; Francisco *et al*, 2013). Therefore we will make no further comments on this issue. The second version of the “export-driven protein import model” hypothesizes that monoubiquitination/export of the shuttling receptors is mechanistically linked to cargo translocation across the peroxisome membrane. In other words, in the absence of monoubiquitination/export of the receptors no cargo translocation can occur. The data obtained in this work, together with findings previously obtained in our laboratory for PTS2 proteins (Alencastre *et al*, 2009), clearly demonstrate that this is not the case, at least for the mammalian PIM.

In summary, the results presented here suggest that translocation of a PTS1 protein across the organelle membrane, including its release into the peroxisomal matrix, occurs downstream of the docking step and upstream of PEX5 ubiquitination, concomitantly with the insertion of the receptor into the DTM. These findings provide a hitherto missing cargo-centered perspective to support the model proposed by our

group, in which PEX5, besides working as a soluble receptor, also functions as a translocator pushing cargo proteins across the peroxisomal membrane as it gets inserted into the peroxisomal docking/translocation machinery (see Figure 20).

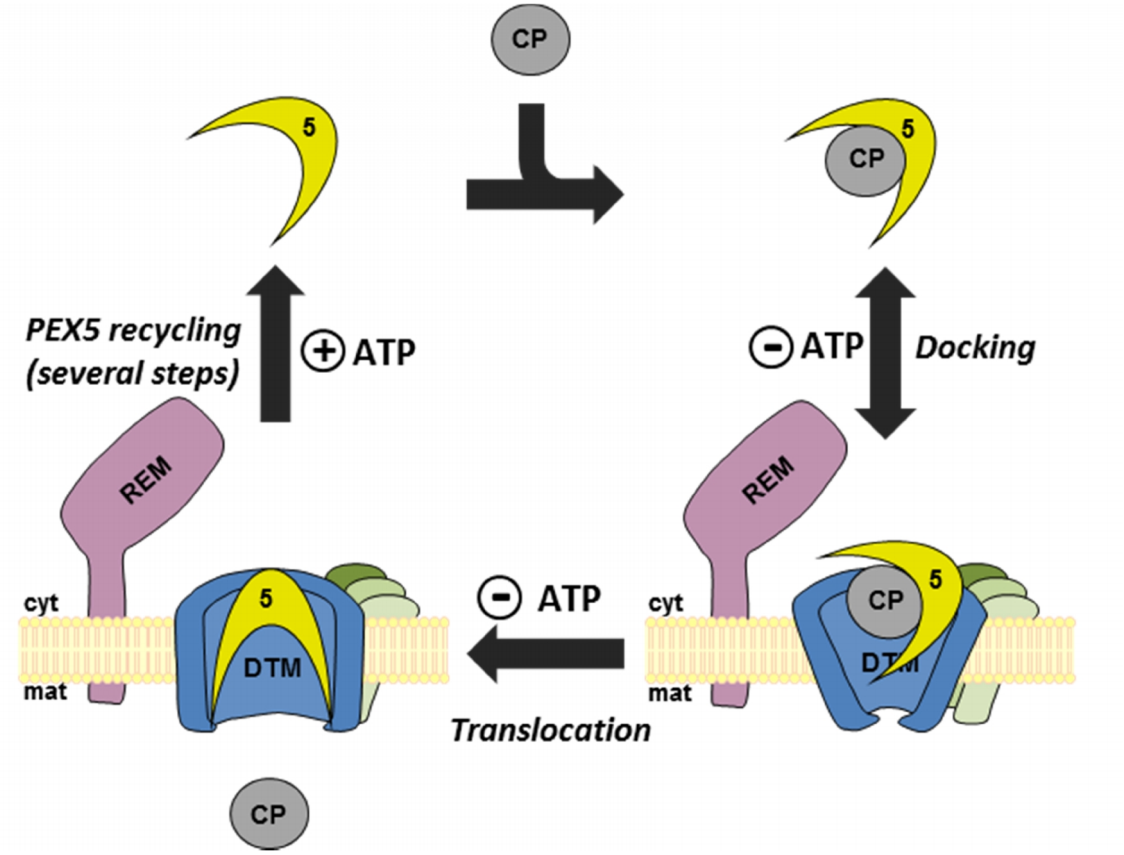


Figure 20. Working model for the PEX5-mediated protein import pathway. After binding a PTS1 protein in the cytosol, PEX5 docks at the DTM in a reversible manner. PEX5 then becomes inserted into the DTM pushing the cargo protein across the organelle membrane. In contrast to PEX5 recycling, which includes monoubiquitination and PEX1/PEX6-catalyzed extraction of the receptor from the DTM, the docking and translocation steps do not require cytosolic ATP.

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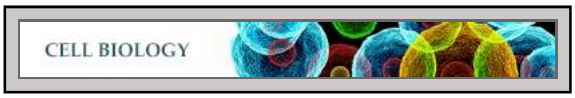
7. PUBLICATIONS

Cell Biology:



A Cargo-centered Perspective on the PEX5 Receptor-mediated Peroxisomal Protein Import Pathway

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A Cargo-centered Perspective on the PEX5 Receptor-mediated Peroxisomal Protein Import Pathway*

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Background: How the soluble receptor PEX5 delivers its cargoes to the peroxisome remains largely unknown.
Results: Cargo translocation occurs after docking of the receptor at the peroxisome and before any ATP-dependent step.
Conclusion: Translocation is concomitant with PEX5 insertion into the docking/translocation machinery.
Significance: These results support a model in which cargoes are pushed across the peroxisomal membrane by PEX5.

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and post-translationally targeted to the organelle by PEX5, the peroxisomal shuttling receptor. The pathway followed by PEX5 during this process is known with reasonable detail. After recognizing cargo proteins in the cytosol, the receptor interacts with the peroxisomal docking/translocation machinery, where it gets inserted; PEX5 is then monoubiquitinated, extracted back to the cytosol and, finally, deubiquitinated. However, despite this information, the exact step of this pathway where cargo proteins are translocated across the organelle membrane is still ill-defined. In this work, we used an *in vitro* import system to characterize the translocation mechanism of a matrix protein possessing a type 1 targeting signal. Our results suggest that translocation of proteins across the organelle membrane occurs downstream of a reversible docking step and upstream of the first cytosolic ATP-dependent step (*i.e.* before ubiquitination of PEX5), concomitantly with the insertion of the receptor into the docking/translocation machinery.

Peroxisomal matrix proteins are encoded by nuclear genes and synthesized on free ribosomes in the cytosol (1). Their sorting to the organelle relies on one of two types of peroxisomal targeting signals (PTS).[5] The PTS type 1 (PTS1) consists of a conserved tripeptide, usually with the sequence S-K-L, present at the C termini of the vast majority of peroxisomal matrix proteins (2, 3). The PTS2 is a degenerated nonapeptide located at the N terminus of only a small number of proteins (4–6). Targeting of newly synthesized matrix proteins to the organelle requires a complex machinery comprising both cytosolic and peroxisomal membrane proteins. A central component of this machinery is PEX5, a monomeric 70-kDa protein rich in intrinsically disordered domains (7–9), which *in vivo* displays a dual subcellular localization, cytosolic and peroxisomal (10). Its function is to carry newly synthesized proteins from the cytosol to the peroxisome, thus working as a shuttling receptor (10). PEX5 interacts directly with PTS1 proteins. This interaction is mediated mainly by the PTS1 of the cargo protein on one side and the tetratricopeptide repeats (TPRs) present in the C-terminal half of PEX5 on the other, but other regions of the cargo protein and other domains of PEX5 are also involved (11–16). Interestingly, recent data suggest that PEX5 may also act as a chaperone at least for some PTS1 proteins (11). In mammals, plants, and many other organisms, PEX5 is also in charge of transporting PTS2 proteins to the peroxisome (17–20). In this case, however, the interaction is not direct but rather mediated by the adaptor protein PEX7 (5, 21, 22).

According to current models (23–26), after binding newly synthesized matrix proteins in the cytosol, PEX5 interacts with the peroxisomal docking/translocation module (DTM), a multisubunit protein assembly comprising five core components: PEX13 and PEX14 and the RING finger peroxins PEX2, PEX10, and PEX12 (27–29). This interaction results in the insertion of PEX5 into the DTM, with PEX5 acquiring a transmembrane topology (Refs. 30 and 31; see also "Discussion"). PEX5 is then monoubiquitinated at a conserved cysteine residue (32, 33), a modification required for the next step of the pathway, the ATP-dependent extraction of monoubiquitinated PEX5 back

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[5] The abbreviations used are: PTS, peroxisomal targeting signals; PTS1, PTS type 1; PTS2, PTS type 2; PNS, postnuclear supernatants; TPR, tetratricopeptide repeat; DTM, peroxisomal docking/translocation module; SCPx, sterol carrier protein x; DECR2, 2,4-dienoyl CoA reductase; Ub, ubiquitin; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

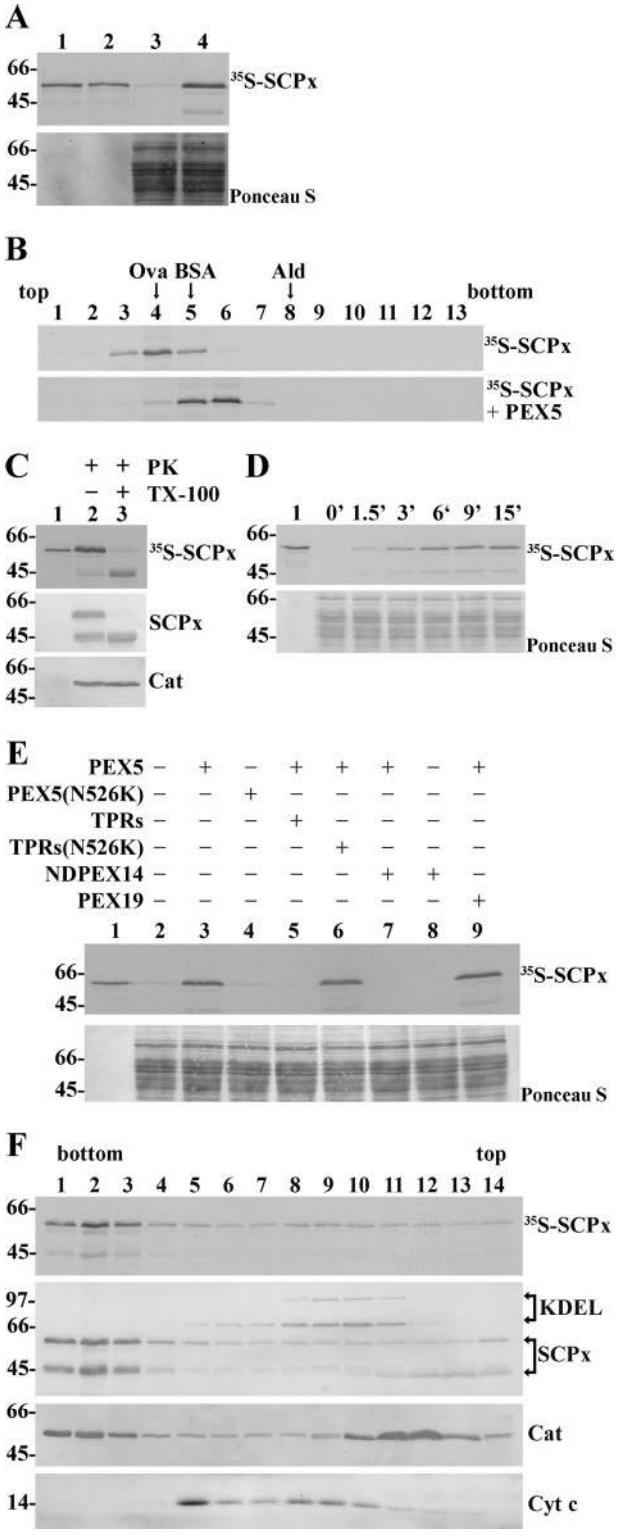

FIGURE 1. **[^{35}S]SCPx is specifically imported into peroxisomes *in vitro*.** *A*, preincubation of [^{35}S]SCPx with recombinant PEX5 improves its *in vitro* import efficiency. Two chemically identical import reactions were assembled, differing solely in the step of the protocol where recombinant PEX5 was added. In one reaction (*lane 3*), [^{35}S]SCPx was preincubated in the absence of PEX5 and added to a PNS in ATP-containing import buffer. After the addition of recombinant PEX5, the reaction was incubated for 15 min at 37 °C. In the other reaction (*lane 4*), [^{35}S]SCPx was preincubated with recombinant PEX5, added to a PNS in the same buffer, and incubated under the same conditions. Proteinase K-treated organelles were then subjected to SDS-PAGE/Western blotting/autoradiography. *Lanes 1* and *2* contain 5% of the preincubated [^{35}S]SCPx proteins used in the assays shown in *lanes 3* and *4*, respectively. The autoradiograph (*upper panel*) and the Ponceau S-stained membrane (*lower panel*) are shown. *B*, *in vitro* synthesized [^{35}S]SCPx interacts with PEX5. [^{35}S]SCPx was preincubated for 30 min at room temperature in the absence or presence of 1 μM recombinant PEX5, as indicated. After adding a mixture of protein standards, the samples were subjected to sucrose gradient centrifugation. After fractionation, equivalent aliquots were subjected to SDS-PAGE/Western blotting. Note that the sedimentation coefficient of [^{35}S]SCPx increases in the presence of PEX5, indicating that the two proteins interact. Autoradiographs are shown. Protein standards used were: ovalbumin (*Ova*; 45 kDa), bovine serum albumin (*BSA*; 66 kDa), and aldolase (*Ald*; 140 kDa). *C*, organelles from an *in vitro* import reaction were resuspended in import buffer and treated with proteinase K (*PK*) in the absence (*lane 2*) or presence of Triton X-100 (*TX-100*; *lane 3*). Samples were analyzed as in *A*. The behaviors of endogenous SCPx and catalase (*Cat*) are shown. *D*, [^{35}S]SCPx preincubated with recombinant PEX5 was subjected to a standard import assay. Aliquots of the reaction were withdrawn at the indicated time points, treated with proteinase K, and processed for SDS-PAGE/autoradiography (*upper panel*). The Ponceau S-stained membrane is also shown (*lower panel*). *E*, [^{35}S]SCPx was preincubated in the absence (−) or presence (+) of the indicated recombinant proteins. Protein mixtures were then subjected to standard import assays and analyzed as described above. Note that PEX19 is involved in a different aspect of peroxisomal biogenesis (59) and was used here just as a negative control. *F*, a protease-treated import reaction was subjected to Nycodenz gradient centrifugation. The behaviors of [^{35}S]SCPx, endogenous SCPx, catalase (*Cat*), cytochrome *c* (*Cyt c*; a mitochondrial marker), and two endoplasmic reticulum proteins (KDEL; recognizes GRP72 and GRP98) are shown. The fraction of catalase detected at the top of the gradient represents mostly catalase that has leaked from peroxisomes during PNS preparation. Unlike soluble SCPx, soluble mouse catalase is quite resistant to proteinase K (see *C*). *Lanes 1* in *C*, *D*, and *E*, 5% of the [^{35}S]SCPx protein used in each reaction. *Numbers to the left* indicate the molecular masses of protein standards in kDa.

to the cytosol (32, 34). This step is catalyzed by the receptor export module, a protein complex comprising the two ATPases, PEX1 and PEX6, and their membrane anchor, PEX26 (35, 36). Finally, monoubiquitinated PEX5 is deubiquitinated probably by a combination of enzymatic and nonenzymatic mechanisms (37–39).

As is evident from the description above, our knowledge of the pathway followed by PEX5 during the protein import process is reasonably detailed. The same, however, cannot be stated regarding the cargo proteins themselves. Indeed, experimental data addressing crucial aspects of this protein import pathway, such as the timing of the translocation step, are still very scarce. In an attempt to fill in this gap, we have recently characterized the import mechanism of pre-thiolase, a PTS2 protein. The data suggest that translocation of pre-thiolase across the peroxisomal membrane occurs before ubiquitination of DTM-embedded PEX5, presumably during insertion of the receptor into the DTM (40). However, whether this is a unique feature of PTS2 proteins emerging from the participation of PEX7 in this process (see also "Discussion"), or a general property of the peroxisomal protein import machinery, remained unknown. In this work, we characterized the import mechanism of sterol carrier protein x (SCPx), a peroxisomal matrix protein containing a canonical PTS1 (41). SCPx is a 59-kDa protein, homodimeric in its native state, which *in vivo* is partially cleaved in the peroxisomal matrix into a 46-kDa polypeptide and a 13-kDa C-terminal fragment (reviewed in Ref. 42). The data suggest that the import of this protein, including its release into the peroxisome matrix, occurs upstream of the PEX5 ubiquitination step, concomitantly with insertion of the receptor into the DTM.

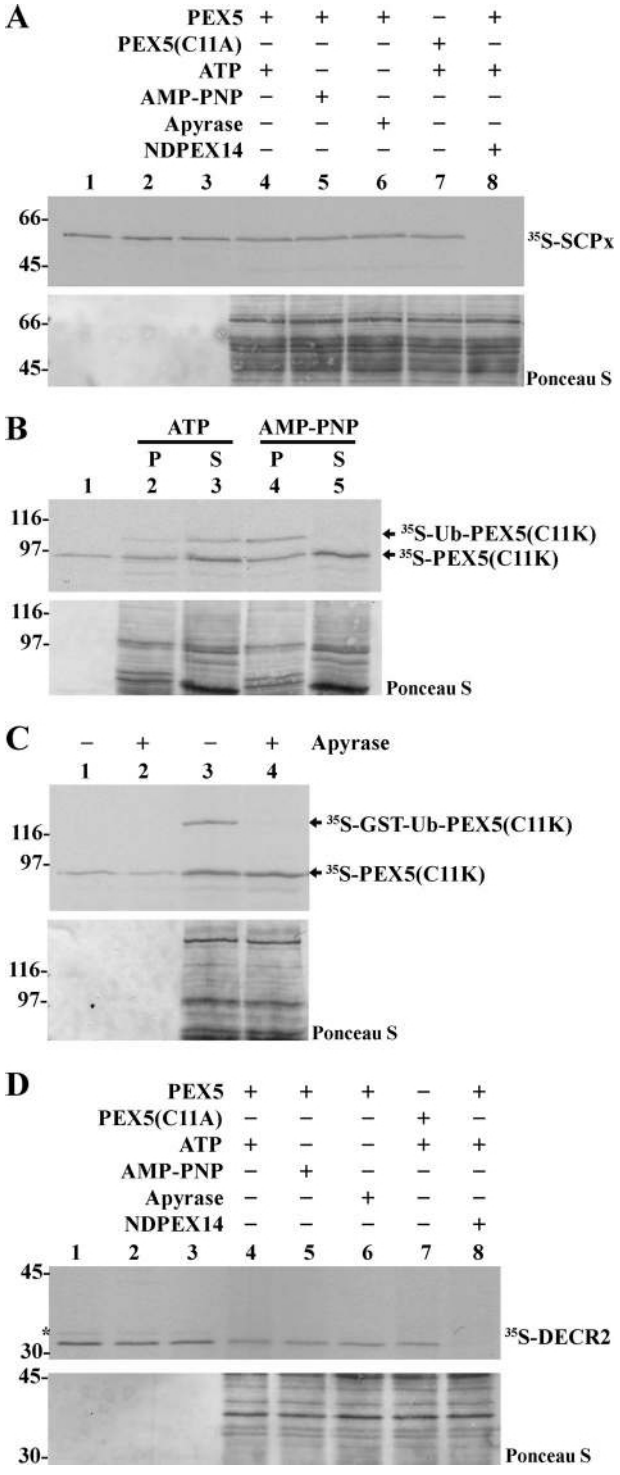

FIGURE 2. **Import of a PTS1 protein into peroxisomes does not require cytosolic ATP.** *A*, [^{35}S]SCPx was preincubated in the presence of either recombinant PEX5 (*lanes 1* and *2*) or PEX5(C11A) (*lane 3*). An aliquot of the PEX5-containing [^{35}S]SCPx was further treated with apyrase (*lane 2*). These samples were then subjected to import assays, as follows: *lane 4*, assay containing ATP and [^{35}S]SCPx preincubated with PEX5; *lane 5*, the same as in *lane 4* but in the presence of AMP-PNP instead of ATP; *lane 6*, assay containing [^{35}S]SCPx preincubated with PEX5 and PNS, both pretreated with apyrase; *lane 7*, import assay containing ATP and [^{35}S]SCPx preincubated with PEX5(C11A); *lane 8*, the same as in *lane 4* but also containing recombinant NDPEX14. Samples were processed as described in the legend for Fig. 1*A*. *Lanes 1–3*, 5% of the [^{35}S]SCPx samples used in the assays. *B*, ^{35}S-labeled PEX5(C11K) was subjected to *in vitro* import reactions containing ubiquitin aldehyde and either ATP (*lanes 2* and *3*) or AMP-PNP (*lanes 4* and *5*). After 7 min at 37 °C, the import reactions were centrifuged to obtain an organelle pellet (*P*) and a supernatant (*S*), and both fractions were analyzed by SDS-PAGE/Western blotting. Note that AMP-PNP allows PEX5(C11K) ubiquitination but not its export into the soluble phase of the import reaction (39). *C*, ^{35}S-labeled PEX5(C11K) was incubated in the absence (−) or presence (+) of apyrase (*lanes 1* and *2*, respectively). The first of these samples (minus apyrase) was subjected to an *in vitro* import assay in the presence of ATP (*lane 3*); the apyrase-treated ^{35}S-labeled PEX5(C11K) was subjected to an import assay using apyrase-treated PNS (*lane 4*). Both reactions also contained 15 μM GST-Ub. The organelles were then isolated by centrifugation and analyzed by SDS-PAGE/Western blotting/autoradiography. Note that GST-Ub is efficiently used by the machinery that monoubiquitinates PEX5 but, in contrast to ubiquitin, results in a PEX5 species that is not exported from the DTM (32, 38). Also, monoubiquitinated PEX5(C11K) is more stable than monoubiquitinated PEX5 upon SDS-PAGE. This property increases the sensitivity of the ubiquitination assays (38). *D*, [^{35}S]DECR2 was subjected to import assays exactly as described in *A* for [^{35}S]SCPx. The *asterisk* indicates an unspecific radiolabeled band produced during the *in vitro* translation reaction. *Lane 1* in *B* and *lanes 1* and *2* in *C*, 5% of the ^{35}S-labeled PEX5(C11K) used in the assays.

EXPERIMENTAL PROCEDURES

Proteins and Plasmids—The recombinant large isoform of human PEX5 (PEX5 (8, 17, 43)), proteins comprising amino acid residues 1–324 and 315–639 of PEX5 (ΔC1PEX5 and TPRs, respectively (7)), PEX5 containing the missense mutation N526K (PEX5(N526K) (44–46)), TPRs with the missense mutation N526K (TPRs(N526K), numbering of full-length PEX5 (44)), a protein comprising the first 80 amino acid residues of human PEX14 (NDPEX14 (7)), PEX19 (47), and a glutathione *S*-transferase-ubiquitin fusion protein (GST-Ub (32)) were obtained as described previously. PEX5 possessing an alanine at position 11 was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), using pQE30-PEX5 as the template (8, 38). pGEM4 (Promega)-based plasmids encoding PEX5 possessing a lysine (PEX5(C11K)) or an alanine residue (PEX5(C11A)) at position 11 were described before (38). The cDNA encoding SCPx (clone MmCD00313611, PlasmID, Dana Farber/Harvard Cancer Center; (41)) was amplified by PCR using the primers 5′-GCGCCGTCTAGAGCCACCATGC-CTTCTGTCGCTTTG-3′ and 5′-GCCGGCGGTACCTCA-CAGCTTAGCTTTGCCC-3′ and cloned into XbaI/KpnI-digested pGEM4 vector (Promega). The cDNA encoding human 2,4-dienoyl-CoA reductase (DECR2) was obtained from the plasmid pKDN36 (48) (a kind gift of Dr. Marc Fransen from KU Leuven, Belgium) by PCR using the primers 5′-GATA-ATTCTAGAGCCACCATGGCCCAGCCGC-3′ and 5′-CGC-CGTGGTACCCTAGAGCTTAGCAGAGAAGGA-3′. The DNA fragment was digested with XbaI and KpnI and cloned into the XbaI/KpnI-digested pGEM4 vector (Promega). ^{35}S-labeled proteins were synthesized *in vitro* as described before (40).

In Vitro Import Experiments—Mouse liver postnuclear supernatant (PNS) was prepared as described before (49). The reticulocyte lysate containing ^{35}S-labeled SCPx ([^{35}S]SCPx) was diluted 1:10 in import buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 3 mM $MgCl_2$, 20 μM methionine, 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide) and incubated for 15 min at 37 °C in the absence or presence of one or more of the following recombinant proteins: PEX5 or PEX5(N526K) or PEX5(C11A) (30 nM final concentration), TPRs or TPRs(N526K) (10 μM final concentration), and NDPEX14 or PEX19 (20 μM final concentration). In a standard import reaction (100 μl final volume), 10 μl of the

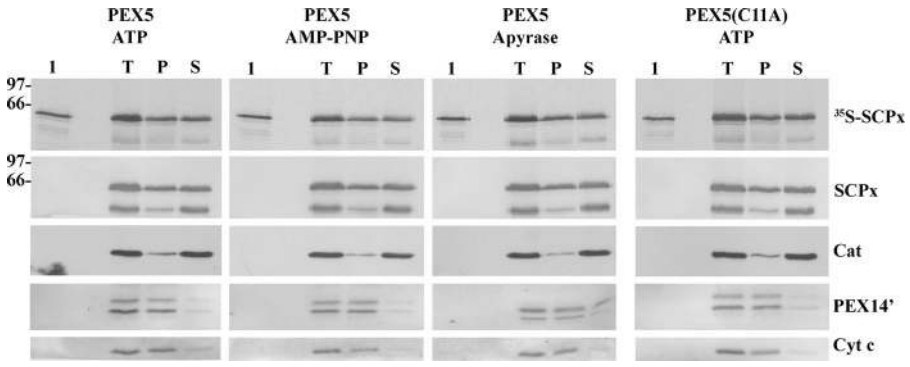

FIGURE 3. ***In vitro* imported [^{35}S]SCPx behaves as endogenous SCPx upon fractionation of peroxisomes.** [^{35}S]SCPx preincubated with the indicated PEX5 proteins was subjected to *in vitro* import assays under different energetic conditions, as specified. At the end of the import reaction, the organelles were treated with proteinase K, isolated by centrifugation, and sonicated. One-half of these samples was kept on ice (*lanes T*), whereas the other half was ultracentrifuged to obtain membrane (*P*) and soluble (*S*) fractions. Samples were analyzed by SDS-PAGE/Western blotting/autoradiography. The behaviors of endogenous SCPx, catalase (*Cat*), PEX14, and cytochrome *c* (*Cyt c*) are also shown. Note that PEX14 is converted into small 16–18-kDa fragments (*PEX14'*) upon proteinase K treatment (see also Ref. 62). *Lane 1*, 5% of the [^{35}S]SCPx protein used in each reaction.

diluted lysate were added to 400 μg of PNS protein that had been primed for import (incubation for 5 min at 37 °C in import buffer containing 0.3 mM ATP; see Refs. 40 and 50). Import assays also contained 2 mM glutathione and, as indicated, ATP (3 mM), AMP-PNP (3 mM), bovine ubiquitin (15 μM), GST-Ub (15 μM), or ubiquitin aldehyde (3 μM). In the apyrase experiments, both the diluted lysate and the PNS in import buffer were incubated at 37 °C with apyrase (20 units/ml, Grade VII, Sigma) for 5 and 2 min, respectively, before starting the import assay. When comparing import efficiencies of SCPx under different energetic conditions, import assays were performed for just 7 min to minimize differences induced by the time-dependent occupation of the DTM by PEX5 (40). After import, samples were subjected to proteinase K digestion (400 μg/ml, 40 min on ice). Processing of organelles for SDS-PAGE/autoradiography was done as described before (40). In some experiments, organelles were isolated by centrifugation, resuspended in import buffer, and subjected to proteinase K digestion (50 μg/ml) in the absence or presence of 1% (w/v) Triton X-100. Sonication and fractionation of organelles were done exactly as described (40).

Nycodenz and Centrifugation of Sucrose Gradients—Nycodenz gradient centrifugation of protease-treated import reactions was done as described (47) except that a Nycodenz gradient comprising 1.5 ml of 45% (w/v), 7 ml of 28% (w/v), 2 ml of 25% (w/v), and 2 ml of 20% (w/v) Nycodenz in 5 mM MOPS-KOH, pH 7.2, and 1 mM EDTA-NaOH, pH 8.0, was used and the centrifugation conditions were 59,000 × *g* for 3 h at 4 °C in a vertical rotor (STEPSAVER^{TM} 65V13, Sorvall®). For the sucrose gradient centrifugation analyses, 10 μl of SCPx lysate in 200 μl of a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA-NaOH, pH 8.0, and 1 mM DTT, supplemented or not with 25 μg of recombinant PEX5, were loaded onto the top of a continuous 5–20% (w/v) sucrose gradient in the same buffer. After centrifugation at 247,000 × *g* for 29 h at 4 °C in an SW41 rotor (Beckman), 13 aliquots were collected from the bottom of the tube. Ovalbumin (3.6 S), bovine serum albumin (4.3 S), and aldolase (7.4 S), were used as internal sedimentation coefficient standards.

Antibodies—The antibodies directed to SCPx (19182-1-AP; ProteinTech^{TM}), catalase (C0979; Sigma), KDEL (ab12223; Abcam), and cytochrome *c* (556433; BD Pharmingen^{TM}) were purchased. The antibody directed to PEX14 was described before (29). Rabbit and mouse antibodies were detected using goat alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies (A9919 and A2429, respectively; Sigma) or goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology).

RESULTS

Our laboratory has been using an *in vitro* import strategy to dissect the mechanism of protein translocation across the mammalian peroxisomal membrane. This strategy comprises three steps: 1) *in vitro* synthesis of a ^{35}S-labeled reporter protein; 2) incubation of the reporter protein with a PNS, a source of peroxisomes and cytosolic components; and 3) treatment of the organelle suspension with a large amount of a protease (such as proteinase K) to degrade nonimported (accessible) reporter protein while preserving the fraction that was imported. This strategy works particularly well when the reporter protein is PEX5 itself (38, 49). In contrast, the import yields obtained using peroxisomal matrix proteins as reporters are in general rather poor. As explained in detail elsewhere (40), a major problem stems from the fact that PNS contains large amounts of soluble PTS1 proteins that have leaked from peroxisomes during tissue homogenization. These soluble proteins compete with the ^{35}S-labeled reporter protein for PEX5 binding, thus resulting in low import yields.

We found that the amounts of protease-protected [^{35}S]SCPx in *in vitro* import assays can be dramatically improved by preincubating this PTS1 protein with recombinant PEX5 before proceeding with the import reaction (Fig. 1*A*, compare *lanes 3* and *4*). Possibly, this step allows the reporter protein to form a complex with PEX5 (Fig. 1*B*) with no competition from the PTS1 proteins present in the PNS. We note that [^{35}S]SCPx is extremely susceptible to proteinase K, a property that is not altered by the preincubation step with PEX5 nor by the simple presence of PNS during the protease treatment (*e.g.* see the temperature dependence experiments below). Actually, the protease-resistant status of [^{35}S]SCPx subjected to import assays vanishes when the protease treatment is made in the presence of the mild detergent Triton X-100 (Fig. 1*C*, *upper panel*), thus suggesting that its protease resistance reflects a

protection effect exerted by a lipid membrane, as is in fact the case for endogenous SCPx (Fig. 1*C*, *middle panel*).

Several experiments were performed to test the specificity of this *in vitro* import system. Acquisition of a protease-protected status by [^{35}S]SCPx is time- (Fig. 1*D*) and temperature-dependent (see below). Furthermore, no protease-protected [^{35}S]SCPx was obtained when PEX5 was replaced by a PEX5 mutant protein that is unable to bind PTS1 proteins efficiently (PEX5(N526K) (45, 46)) (Fig. 1*E*, *lane 4*). The same result was obtained when [^{35}S]SCPx was preincubated with PEX5 plus a molar excess of a protein comprising the PTS1-binding domain of PEX5 (TPRs; Fig. 1*E*, *lane 5*). The latter protein can still bind PTS1 proteins efficiently but lacks the N-terminal domain of PEX5 required for a productive interaction with the peroxisomal DTM (51). A mutant version of TPRs carrying the N526K mutation (TPRs(N526K)) has no such effect (*lane 6*). Finally, no protease-resistant SCPx was observed when the reporter protein was preincubated with PEX5 plus a recombinant protein comprising the N-terminal domain of PEX14, a component of the DTM (*lane 7*). This domain of PEX14 binds with high affinity to the so-called diaromatic motifs present in the N-terminal half of PEX5 (52), which are essential for PEX5 function (53).

To confirm that the membrane-bound organelle to which PEX5 targets SCPx is in fact the peroxisome, a protease-treated import reaction was subjected to Nycodenz gradient centrifugation (54). As shown in Fig. 1*F*, most *in vitro* imported [^{35}S]SCPx was found in fractions 1–3, the region of the gradient containing intact peroxisomes. Taken together, these data show that [^{35}S]SCPx can be efficiently imported into peroxisomes *in vitro*.

Having established the robustness and specificity of this *in vitro* import system, we then asked whether import of SCPx requires hydrolysis of cytosolic ATP. Two different strategies were used to address this question. In the first, a PNS that had been primed for import in the presence of 0.3 mM ATP (see "Experimental Procedures" for details) was used in an import reaction containing 3 mM AMP-PNP. AMP-PNP is a potent inhibitor of ATPases cleaving the bond between the β- and γ-phosphate groups of ATP. Note that ubiquitination of PEX5 at the DTM still occurs in the presence of AMP-PNP because the ubiquitin-activating enzyme uses this ATP analog quite efficiently (39, 55). However, export of monoubiquitinated PEX5 from the DTM to the cytosol, a process catalyzed by the ATPases PEX1/PEX6, is completely blocked by AMP-PNP (39). As shown in Fig. 2*A*, the import efficiencies of [^{35}S]SCPx in reactions supplemented with either ATP (*lane 4*) or AMP-PNP (*lane 5*) are essentially the same. Thus, a 10-fold molar excess of AMP-PNP over ATP does not result in an inhibition of SCPx import, although export of monoubiquitinated PEX5 is blocked under these conditions (Fig. 2*B*), as expected (39).

In the second strategy, both [^{35}S]SCPx and a primed PNS were treated with apyrase, an enzyme that hydrolyzes ATP and other NTPs (56), before the import reaction. As shown in Fig. 2*A*, the import efficiency of SCPx was not decreased by the apyrase treatment (compare *lanes 4* and *6*). A control experiment shows that the apyrase treatment efficiently depletes ATP from the reactions because ubiquitination of PEX5 was no longer observed under these conditions (Fig. 2*C*), as described before (40). Taken together, these results strongly suggest that import of SCPx into peroxisomes requires neither monoubiquitination of PEX5 nor even hydrolysis of cytosolic ATP. Additional evidence supporting the first of these conclusions was obtained when [^{35}S]SCPx was preincubated with recombinant PEX5(C11A) instead of PEX5. PEX5(C11A) possesses an alanine at position 11, a non-ubiquitinatable amino acid residue. Therefore, this mutant PEX5 is not monoubiquitinated at the DTM and, consequently, it is not exported back to the cytosol

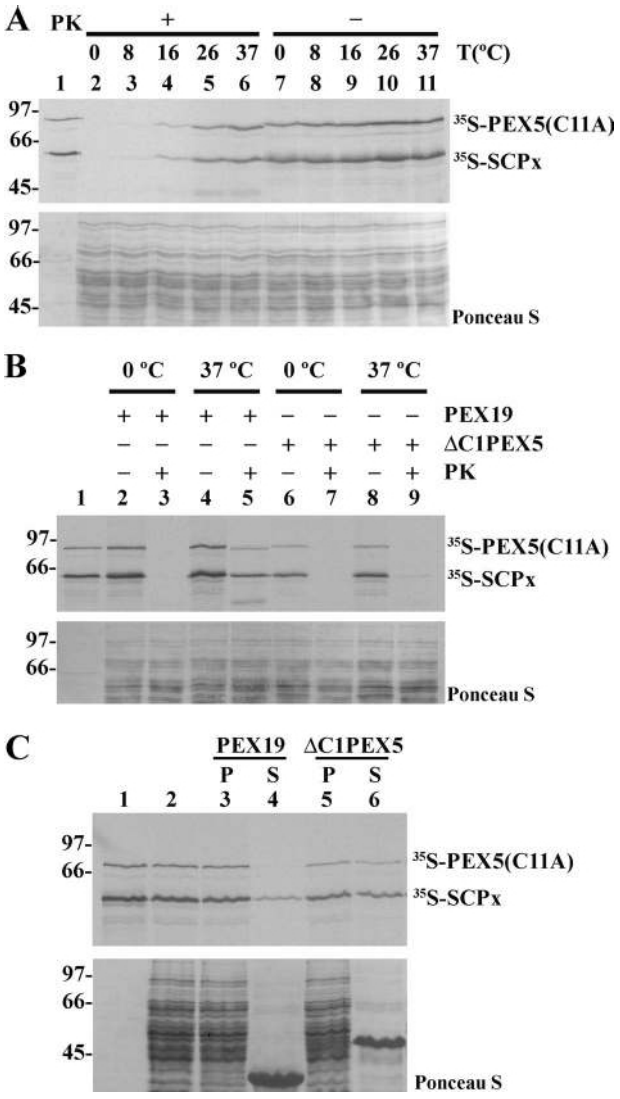

FIGURE 4. **Temperature dependence of the docking/insertion of PEX5 into the DTM and SCPx import.** *A*, [^{35}S]SCPx preincubated with a mixture of recombinant and ^{35}S-labeled PEX5(C11A) was subjected to import assays at different temperatures. After 15 min, the samples were halved and treated (*lanes* +) or not (*lanes* −) with proteinase K (*PK*). The organelles were analyzed by SDS-PAGE/autoradiography (*upper panel*). The Ponceau S-stained membrane is also shown (*lower panel*). *B*, [^{35}S]SCPx preincubated as described above was subjected to import assays at 0 or 37 °C in the presence of either recombinant ΔC1PEX5 or PEX19 (5 μM each), as indicated. Protease-treated and untreated organelles were then analyzed as above. *C*, organelles from an import assay performed at 0 °C (*lane 2*) were resuspended in import buffer containing either recombinant ΔC1PEX5 or PEX19, incubated for 15 min, and reisolated by centrifugation. Organelle pellets (*P*) (*lanes 3* and *5*) and the corresponding supernatants (*S*) (*lanes 4* and *6*) were analyzed as above. *Lanes 1* in *A*, *B*, and C, 5% of the radiolabeled proteins used in the assays.

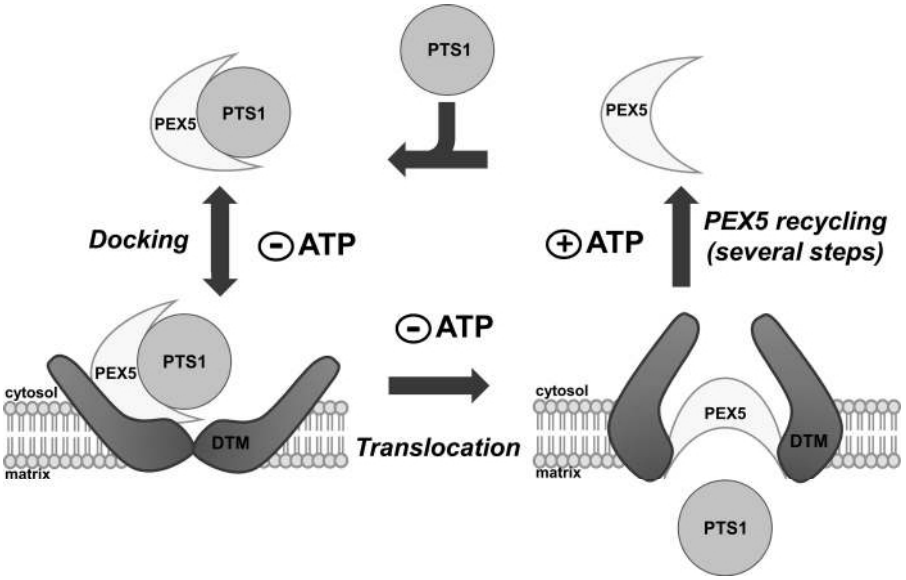

FIGURE 5. **Working model for the PEX5-mediated protein import pathway.** After binding a PTS1 protein in the cytosol, PEX5 docks at the DTM in a reversible manner. PEX5 then becomes inserted into the DTM, pushing the cargo protein across the organelle membrane. In contrast to PEX5 recycling, which includes monoubiquitination and PEX1/PEX6-catalyzed extraction of the receptor from the DTM, the docking and translocation steps do not require cytosolic ATP.

(38). As shown in Fig. 2*A*, PEX5(C11A) is as efficient as PEX5 in these assays (compare *lanes 4* and *7*).

The ability to be imported into peroxisomes in a PEX5 monoubiquitination- and cytosolic ATP-independent manner is not a particularity of SCPx. Indeed, exactly the same results were obtained when [^{35}S]DECR2, another peroxisomal matrix PTS1 protein, was used in these assays (Fig. 2*D*).

The experiments above indicate that [^{35}S]SCPx acquires a protease-protected peroxisomal location in a process that requires PEX5 but not hydrolysis of cytosolic ATP. However, it remained unclear whether the protease-protected [^{35}S]SCPx detected in those experiments represents a protein that was already completely translocated into the peroxisomal matrix or a species that is still associated with the DTM. To address this issue, [^{35}S]SCPx preincubated with either PEX5 or PEX5(C11A) was subjected to import assays in the presence of ATP or AMP-PNP or apyrase, as specified in Fig. 3. After protease treatment, the organelles were isolated by centrifugation, disrupted by sonication, and ultracentrifuged to obtain membrane and soluble fractions. The efficiency of the fractionation procedure was assessed by Western blotting using antibodies directed to PEX14 (a peroxisomal intrinsic membrane protein), cytochrome *c* (a peripheral membrane mitochondrial protein), and catalase (a soluble peroxisomal matrix protein). As shown in Fig. 3, a major fraction of endogenous SCPx was found in the soluble fraction, although some protein was also recovered in the membrane pellet. The dual behavior of SCPx might be related to the fact that the C-terminal domain of SCPx binds membrane lipids (57). Importantly, and regardless of the experimental conditions used in the import assays, *in vitro* imported [^{35}S]SCPx displayed exactly the behavior of endogenous SCPx, thus suggesting that it represents a species that was already translocated into the matrix of the organelle.

The data presented above suggest that translocation of SCPx across the peroxisomal membrane occurs upstream of PEX5 monoubiquitination step. According to current models, there are only two events occurring at the peroxisome before this step: 1) docking of the PEX5-cargo protein complex at the DTM and 2) insertion of PEX5 into this machinery. We note, however, that although there is abundant experimental evidence supporting the concept that PEX5 becomes inserted into the DTM (30, 31, 35, 50), data regarding the docking step itself are still very limited. Actually, it is presently unknown whether such a step really exists in a mechanistic sense or whether docking and insertion of PEX5 into the DTM are simply the beginning and the end of a single step. To clarify this issue, we explored the fact that insertion of PEX5 into the DTM is inhibited at low temperatures (58) and asked whether docking of the PEX5-cargo protein complex can still occur under those conditions. Fig. 4 shows *in vitro* import assays where [^{35}S]SCPx was preincubated with a mixture of recombinant PEX5(C11A) and ^{35}S-labeled PEX5(C11A), thus allowing us to monitor the behavior of the cargo and the receptor simultaneously. As expected, insertion of PEX5(C11A) into the DTM (as assessed by the acquisition of a protease-resistant status (49)) does not occur at low temperatures (Fig. 4*A*, compare *lanes 2* and *3* with *lanes 4–6*). Importantly, import of SCPx displays the same temperature dependence profile. When the protease treatment was omitted, both ^{35}S-labeled PEX5(C11A) and ^{35}S-labeled SCPx were found in the organelle fractions even in import reactions performed at low temperatures (Fig. 4*A*, *lanes 7* and *8*). A considerable fraction of these proteins is specifically adsorbed to the peroxisome because their amounts in assays containing recombinant ΔC1PEX5, a PEX5 protein that lacks the PTS1-binding domain but that is still competent in entering the DTM (30, 39), are smaller than those observed in the presence of PEX19, a protein involved in another aspect of peroxisome biogenesis (59) and used here as a negative control (Fig. 4*B*, compare *lanes 2* and *4* with *6* and *8*, respectively). A similar competition phenomenon was observed when organelles isolated from an import assay performed at 0 °C were washed in buffer containing ΔC1PEX5 (Fig. 4*C*, compare *lanes 3* and *4* with *lanes 5* and *6*,

respectively). Apparently, both ^{35}S-labeled PEX5(C11A) and ^{35}S-labeled SCPx can interact in a specific and reversible manner with peroxisomes. Taken together, these results provide evidence for the existence of a mechanistically distinct docking step of the PEX5-cargo protein at the DTM and suggest that import of SCPx occurs concomitantly with insertion of PEX5 into the DTM.

DISCUSSION

We have previously shown that cytosolic PEX5 becomes transiently inserted into the peroxisomal membrane DTM in a process that is cargo protein-dependent but independent of cytosolic ATP (30, 31, 50). Those findings together with data suggesting that DTM-embedded PEX5 adopts a transmembrane topology exposing a major fraction of its polypeptide chain into the lumen of the organelle (31, 49) led us to propose that the PEX5-mediated translocation of cargo proteins across the peroxisomal membrane is an ATP-independent event that occurs concomitantly with insertion of PEX5 into the DTM (50, 60). According to this model, the driving force for the protein translocation step resides in the strong protein-protein interactions that are established between PEX5 on one side and components of the DTM on the other; ATP hydrolysis is necessary only at later steps, to extract PEX5 from the DTM, thus resetting the protein transport system (see Ref. 23 and references cited therein). Although essentially all the data on PEX5 collected since then using several experimental systems are compatible with such a model (35, 36), the fact remained that no direct evidence showing ATP-independent import of a peroxisomal matrix protein was available for many years. This gap in experimental evidence was partially filled in by recent findings showing that translocation of pre-thiolase (a PTS2 protein) across the peroxisomal membrane occurs before monoubiquitination of PEX5 at the DTM, in a cytosolic ATP-independent manner (40). However, considering that PTS2 proteins are targeted to the peroxisome by a PEX5-PEX7 protein complex and that there are some data suggesting that PEX7 may actually enter the organelle matrix together with PTS2 proteins (61), it remained unclear whether the mechanistic data gathered for PTS2 proteins were also valid for PTS1 proteins.

The data presented here indicate that neither a nonhydrolyzable ATP analog nor the depletion of ATP from import reactions affects the PEX5-mediated peroxisomal import of PTS1 proteins. These findings, besides indicating that import of PTS1 proteins does not require cytosolic ATP, also imply that the import process occurs before PEX5 monoubiquitination. Indeed, the import efficiencies and behaviors of [^{35}S]SCPx upon the peroxisome fractionation experiments were the same when using recombinant PEX5 or PEX5(C11A). Thus, contrary to previous hypotheses (23, 33), neither ubiquitination of PEX5 at the DTM nor the ATP-dependent extraction of monoubiquitinated PEX5 from the DTM plays a role in the cargo protein translocation steps.

The conclusion that import of a PTS1 protein occurs upstream of PEX5 monoubiquitination immediately indicates that translocation of the cargo protein across the organelle membrane occurs during the docking/insertion of PEX5 at/into the DTM. By performing import reactions at several temperatures, it was possible to resolve these two steps. We found that docking of both PEX5 and SCPx at the organelle surface can still occur at 0 °C; insertion of PEX5 into the DTM and import of SCPx into peroxisomes, however, were only detected at higher temperatures.

In summary, the results presented here suggest that translocation of a PTS1 protein across the organelle membrane occurs downstream of the docking step and upstream of PEX5 ubiquitination, concomitantly with the insertion of the receptor into the DTM. These findings provide a hitherto missing cargo-centered perspective to support a model in which PEX5, besides working as a soluble receptor, also functions as a translocator pushing cargo proteins across the peroxisomal membrane as it gets inserted into the peroxisomal docking/translocation machinery (Fig. 5).

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Review

Ubiquitin in the peroxisomal protein import pathway

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ABSTRACT

PEX5 is the shuttling receptor for newly synthesized peroxisomal matrix proteins. Alone, or with the help of an adaptor protein, this receptor binds peroxisomal matrix proteins in the cytosol and transports them to the peroxisomal membrane docking/translocation module (DTM). The interaction between cargo-loaded PEX5 and the DTM ultimately results in its insertion into the DTM with the concomitant translocation of the cargo protein across the organelle membrane. PEX5 is not consumed in this event; rather it is dislocated back into the cytosol so that it can promote additional rounds of protein transportation. Remarkably, the data collected in recent years indicate that dislocation is preceded by mono-ubiquitination of PEX5 at a conserved cysteine residue. This mandatory modification is not the only type of ubiquitination occurring at the DTM. Indeed, several findings suggest that defective receptors jamming the DTM are polyubiquitinated and targeted to the proteasome for degradation.

1. Introduction

Covalent attachment of ubiquitin (Ub) to a protein substrate requires an enzymatic cascade comprising three components: 1) an ATP-dependent ubiquitin-activating enzyme (E1); 2) an ubiquitin-conjugating enzyme (E2); and 3) an ubiquitin ligase (E3). For many years this post-translational modification was best known as a signal leading to protein degradation at the proteasome [1]. Presently, however, it is clear that ubiquitination is also used in a reversible manner to regulate almost all biological pathways in eukaryotic cells [2–4]. This reversibility is ensured by the action of deubiquitinating enzymes (DUBs), a group of proteases that specifically remove the ubiquitin moieties from the modified proteins [5].

The generalized use and complexity of ubiquitination/deubiquitination strategies in the spatiotemporal regulation of biological processes can be easily appreciated by considering just two properties of ubiquitin biology. The first is the overwhelming number of genes encoding proteins dedicated to ubiquitin conjugation/deconjugation that can be found in any eukaryotic organism (mammals have 2 E1s, 40 E2s, more than 600 E3s and approximately 100 DUBs) [6]. The second is that ubiquitination can take many different forms. Indeed, proteins can be modified with a single ubiquitin (monoubiquitination), two or more ubiquitin molecules, each attached to a different amino acid residue (multi-ubiquitination), or with an ubiquitin chain (polyubiquitination). In the latter case, the complexity is further increased because different E2/E3 pairs can build polyubiquitin chains with different topologies. The final outcome of each of these modifications is not the same because the effectors that ultimately recognize and decode these ubiquitin signals are also different [7].

This review focuses on the mechanism of protein sorting into the peroxisome matrix, a biological pathway providing a remarkable example of how ubiquitination is used not just as part of a quality control process but also as one of its intrinsic steps.

Abbreviations: AAA, ATPases associated with diverse cellular activities; DTM, docking/translocation module; DUBs, deubiquitinating enzymes; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; ERAD, Endoplasmic Reticulum-associated degradation; HECT, Homologous to E6-AP C terminus; IBR, in-between-RING; peroxin, protein specifically involved in peroxisome biogenesis; PIM, peroxisomal import machinery; PTS, peroxisome targeting signal; TPRs, tetratricopeptide repeats; RBR, RING–IBR-RING; REM, receptor export module; RING, really interesting new gene; Ub, ubiquitin; Ub-PEX5, mono-ubiquitinated PEX5.

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2. An overview on the peroxisomal import machinery (PIM)

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and post-translationally targeted to the peroxisome via one of two peroxisome targeting signals (PTS) [8]. The majority of them possess a PTS type 1 (PTS1) at their C termini, a conserved tripeptide, usually with the sequence S-K-L [9,10]. Some peroxisome matrix proteins contain instead a PTS2. This is an N-terminal degenerated nonapeptide with the sequence (R/K)-(L/V/I)-X_5-(H/Q)-(L/A), which in higher eukaryotes is cleaved upon import [11–14]. In mammals, plants and many other organisms both PTS1 and PTS2 proteins are transported to the peroxisome by PEX5, the peroxisomal shuttling receptor [15–19]. PEX5 is a monomeric 70-kDa protein rich in intrinsically disordered domains [20–22]. The interaction of PEX5 with PTS1 proteins is mediated by the PTS1 on one side and a PEX5 domain containing seven tetratricopeptide repeats (TPRs) on the other, but other regions of the cargo protein and other domains of PEX5 also contribute to the interaction [23–27]. The PTS2·PEX5 interaction, on the other hand, requires the adaptor protein PEX7 [15,17–19]. The situation in yeasts and fungi is slightly different because their PEX5 proteins lack a PEX7-interacting domain. In these organisms, PTS2 proteins are instead transported to the peroxisome by a protein complex comprising PEX7 and a species-specific peroxin that displays structural/functional similarities to the N-terminal half of mammalian PEX5 [12,28,29]. These species-specific peroxins (*i.e.*, PEX18, PEX20 and PEX21; see Table 1) are here referred to as PEX5-related proteins.

Following cargo recognition in the cytosol, PEX5 interacts with the docking/translocation module (DTM), a multisubunit protein complex of the peroxisomal membrane comprising the core components PEX2, PEX10, PEX12, PEX13 and PEX14 (see Table 1) [30–32]. This interaction ultimately results in the insertion of PEX5 into the DTM with PEX5 adopting a transmembrane topology [33,34]. The presently available data suggest that cargo translocation across the peroxisomal membrane is coupled to the insertion of the receptor into the DTM ([35,36] and unpublished results). Remarkably, none of these steps requires ATP hydrolysis, a finding that led to the proposal that the driving force for the cargo translocation step resides in the strong, essentially irreversible, interactions that PEX5 establishes with components of the DTM [37–40].

Upon release of its cargo, DTM-embedded PEX5 has to be exported to the cytosol so that it can promote additional rounds of protein transportation. Many of the details of this process have been uncovered in recent years. First, PEX5 is monoubiquitinated in an unconventional manner [41,42]. Then, this monoubiquitinated PEX5 species (Ub-PEX5) is extracted from the DTM in an ATP-dependent manner by the receptor export module (REM) [40,41,43]. This is a protein complex comprising the two AAA ATPases, PEX1 and PEX6, and their membrane anchor PEX26 (or PEX15 and APEM9 in *Saccharomyces cerevisiae* and plants, respectively; see Table 1) [44–47]. Finally, the ubiquitin moiety is removed from Ub-PEX5 probably by a combination of enzymatic and non-enzymatic mechanisms thus resetting the protein transport system [48–50].

Table 1
The Peroxisomal Import Machinery (PIM).

PIM Components	Organisms	Properties
Shuttling Receptors	PEX5	M, P, Y, F	Cytosolic/Peroxisomal; IDD/TPRs; PTS1 sorting
	PEX5.PEX7	M, P	Cytosolic/Peroxisomal; PTS2 sorting
	(PEX18/PEX21).PEX7	Y	
	PEX20.PEX7	Y, F	
Docking Translocation Module (DTM)	PEX14	M, P, Y, F	Peroxisomal; coiled-coil
	PEX13	M, P, Y, F	Peroxisomal; SH3
	PEX17	Y	Peroxisomal; coiled-coil
	PEX14/17(PEX33)	F	Peroxisomal; coiled-coil
	PEX8	Y, F	Peroxisomal
	PEX2	M, P, Y, F	Peroxisomal; RING zinc-binding domain
	PEX10	M, P, Y, F	
	PEX12	M, P, Y, F	
Receptor Export Module (REM)	PEX1.PEX6.anchor[#]	M, P, Y, F	Peroxisomal; AAA ATPase
	AWP1	M	Cytosolic; adaptor protein; A20 and AN1 zinc finger domains
Ubiquitin-conjugating enzymes	E2D1/2/3	M	Cytosolic; E2
	PEX4.PEX22	P, Y, F	Peroxisomal; E2
Deubiquitinating enzymes (DUBs)	USP9X	M	Cytosolic
	UBP15	Y	Partially Peroxisomal

Peroxisomal protein import components are organized into functional/structural units. Their subcellular localization and key features, as well as their distribution among different organisms are indicated. M, mammals; P, plants; Y, yeast; F, fungi; IDD, intrinsically disordered domain; TPRs, tetratricopeptide repeats; SH3, Src homology 3 domain; RING, really interesting new gene; AAA, ATPases associated with diverse cellular activities.
[#] PEX1.PEX6-membrane anchor: PEX26 (M, Y, F), APEM9 (P), or PEX15 (Y). PEX8 [30]; PEX17 [96]; PEX14/17 [97]; PEX33 [98].

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Monoubiquitination of PEX5 or PEX5-related peroxins is not the only type of ubiquitination occurring at the DTM. Indeed, a number of studies have revealed that these receptors are also targets of polyubiquitination, a modification that probably reflects the existence of a quality control system [51–54]. The properties of both types of ubiquitination occurring at the DTM are described below.

3. Peroxisomes and the ubiquitin-proteasome system

Although mammalian PEX2, a RING finger peroxin and a core component of the DTM, was one of the first proteins involved in peroxisome biogenesis to be identified [55–57], the link between RING proteins and E3s was not known at the time [58], and, therefore, the connection between peroxisome biogenesis and the ubiquitin pathway was not immediately perceived. The first data pointing to this connection came from the identification of a yeast peroxin, PEX4. Primary structure analysis of this protein revealed an obvious homology with ubiquitin E2s and, indeed, mutation of its catalytic cysteine was sufficient to block peroxisome biogenesis in yeast [59]. Further characterization of PEX4 revealed that this E2 is anchored to the peroxisomal membrane via PEX22, an intrinsic membrane protein. In the absence of PEX22, PEX4 becomes unstable indicating that the two proteins comprise a functional/structural unit [60]. Strikingly, all attempts to identify the mammalian PEX4 and PEX22 counterparts using either genomic or proteomic approaches failed [61–66] (but see Section 4).

Interestingly, the steady-state levels of PEX5 and PEX20 in *Pichia pastoris* strains lacking PEX4 were found to be heavily decreased [53,60,67], a phenomenon that could be reversed by the simultaneous deletion of any of a group of genes encoding components of the DTM [67]. In strains lacking components of the REM the steady-state levels of both receptors were also diminished [53,67]. Apparently, a blockade at late steps of the import pathway induces the degradation of the shuttling receptors. Although no such phenomenon is observed in *S. cerevisiae*, it was shown that a fraction of PEX5 found in mutant strains lacking PEX4, PEX1 or PEX6 is ubiquitinated, a process involving the cytosolic E2s Ubc1, Ubc4 and Ubc5 [51,52,54,60]. Altogether these data led to the proposal that there is an ubiquitin-based quality control system acting on receptors at the DTM that can no longer return to the cytosol using the normal mechanism (see Fig. 1 and following section).

4. Ubiquitination as an intrinsic step of the peroxisomal import pathway

The data described above implicated PEX4 as an important player of the PIM, but did not unveil its mechanistic role. Research on this issue turned out to reveal one of the most interesting aspects of the PIM. The first hints on the function of PEX4 emerged from bioinformatic analyses proposing that the PIM and the Endoplasmic Reticulum-associated degradation (ERAD) machinery display structural/functional similarities [68,69]. In particular, those studies postulated that the role of PEX4 in the PIM should be similar to the one of Ubc1/Ubc6/Ubc7 in the ERAD system, namely, the ubiquitination of a membrane-associated substrate so that it can be recognized and dislocated into the cytosol by AAA ATPases. The substrate in the ERAD system is a misfolded protein *en route* to the proteasome whereas in the PIM the substrate should be DTM-embedded PEX5 or PEX5-related proteins. Indeed, subsequent work using yeast and a mammalian peroxisomal *in vitro* import system provided the experimental evidence to support this hypothesis. Collectively, these studies showed that: 1) yeast PEX4 monoubiquitinates PEX5 at a conserved cysteine residue [42]; 2) mammalian DTM-embedded PEX5 is also monoubiquitinated at the conserved cysteine [41]; and 3) monoubiquitination of yeast and mammalian PEX5 at the DTM is a mandatory step for their subsequent export into the cytosol, a process catalyzed by the ATP-dependent REM [41,43]. These findings also provided the explanation for earlier reports showing that deletion of a small N-terminal domain containing the conserved cysteine residue of human PEX5, or mutation of this cysteine in both *P. pastoris* PEX20 and human PEX5, resulted in proteins that could still enter the DTM but that were no longer substrates for the REM [38,70,71]. Recently, direct evidence showing that *S. cerevisiae* PEX18 and *P. pastoris* PEX20 are indeed modified by this type of unconventional ubiquitination was provided [72,73].

The finding that mammalian and yeast PEX5 are both monoubiquitinated at the DTM was unexpected at the time because, as stated above, mammals lack PEX4 and PEX22. On one hand, it was now obvious that the PIM of yeasts and mammals operate using similar principles, despite significant differences in their protein composition (see Table 1) [30–32]. On the other hand, it was evident that we were still missing components of the mammalian PIM. Using a peroxisome-dependent PEX5 monoubiquitination assay it was found that the long-sought mammalian E2 activity co-fractionated with cytosolic proteins [74]. Actually, a simple low speed centrifugation of a post-nuclear supernatant was sufficient to separate the E2 activity involved in this unconventional ubiquitination from peroxisomes, indicating that contrary to the situation in yeasts/fungi and probably also plants [59,60,66,75], the mammalian E2 enzyme is not stably bound to the peroxisomal membrane. Standard protein purification procedures followed by mass spectrometry led to its identification. Interestingly, not one but rather three different E2s were found in that study. These are the almost identical E2D1, E2D2 and E2D3 (also known as UbcH5a, b and c in humans), a group of multipurpose cytosolic E2 enzymes involved in numerous biological pathways [76,77].

Three of the five core components of the DTM have Zn^{2+}-binding domains. These are PEX2, PEX10 and PEX12, a trio of proteins generally referred to as the “RING peroxins”. However, it must be noted that the typical sequence motif that characterize RING domains is found only in PEX10 from all organisms. Most PEX2 proteins also have this motif but there are some notorious exceptions (*e.g.*, *S. cerevisiae*) [78,79], whereas all PEX12 proteins are completely atypical, lacking several of the eight conserved Zn^{2+}-binding residues found in RING domains. Indeed, the corresponding domain of *S. cerevisiae* PEX12 was recently shown to bind only one Zn^{2+} [78]. Considering that RING domains define the largest class of E3 ubiquitin ligases, it was evident from the very first findings on receptor ubiquitination that the RING peroxins must have a role in these modifications. In agreement with this idea, it has been reported that the Zn^{2+}-binding domains alone have E3 activity in *in vitro* ubiquitination assays [79–81]. Interestingly, although the Zn^{2+}-binding domains of all these proteins are exposed into the cytosol, monoubiquitination of PEX5 and PEX5-related proteins occurs only when these receptors are already embedded in the DTM. On the other hand, insertion of PEX5 into the DTM is not dependent on these peroxins [16,30,67,82]. Thus, the DTM resembles multisubunit E3 ligases, in which substrates are recruited not by the RING proteins themselves but rather by other subunits of the protein complex (see Ref. [39]).

Which of the three RING peroxins of the DTM (if any alone) mediates the unconventional ubiquitination of PEX5 remains unknown. We note that some attempts to address this issue using recombinant peroxin Zn^{2+}-binding domains and PEX5 in *in vitro* ubiquitination assays have been reported [81]. However, no evidence for *bona fide* monoubiquitinated PEX5 was found so far.

An interesting property of the RING peroxins is that the absence of any of these proteins leads to the instability of the other two [30,83,84]. This phenomenon suggests that the three proteins

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comprise a structural unit within the DTM, as is in fact supported by protein purification studies in yeast [30]. Interestingly, recent data suggest that the three RING peroxins may also display a functional interdependence. Indeed, disruption of the RING domain of any of these peroxins leads to a complete loss of both mono- and poly-ubiquitination of *P. pastoris* PEX20 [73]. These important findings suggest that all RING peroxins *en bloc* are required for both types of receptor ubiquitination and raise the appealing possibility that the RING peroxins may be simply modules of a single multi-Zn^{2+}-binding domain ubiquitin ligase. An example of this type of architecture is provided by the RING-in-between-RING(IBR)-RING (RBR) family of ubiquitin ligases, a class of E3s that use a Homologous to E6-AP C terminus (HECT) E3-like mechanism to ubiquitinate a substrate. The catalytic regions of RBR E3s comprise three closely spaced domains: a canonical RING domain (RING1) which serves as the binding platform for the ubiquitin-loaded E2; a Zn^{2+}-binding domain (the so-called IBR) which probably has a structural/regulatory role; and another Zn^{2+}-binding domain, originally named the RING2 domain, which contains the catalytic cysteine [85]. RBR E3 ligases, therefore, provide a remarkable example of how multiple Zn^{2+}-binding domains can be structurally and functionally organized to perform a single function. Considering the *P. pastoris* data referred to above, it is tempting to speculate that a similar, although not necessarily identical, situation will be found for the RING peroxins.

5. Receptor dislocation and deubiquitination

According to current models (see Fig. 1 and Refs. [39,86–88]), there are at least four steps occurring during the transient passage of PEX5 through the peroxisomal DTM before its export into the cytosol: 1) docking; 2) insertion into the DTM/cargo protein translocation; 3) cargo release into the peroxisome matrix; and 4) monoubiquitination. In principle, monoubiquitination of PEX5 could coincide in time with any of the other three steps and even modulate/trigger one of them as was in fact previously proposed for the cargo release step [42]. However, several findings obtained with an *in vitro* peroxisomal import system suggest that this is not the case. Indeed, in the absence of an operating ubiquitin-conjugation cascade PEX5 can still enter the DTM where it acquires the expected transmembrane topology [40,41]; the same is true for PEX5 mutant proteins lacking the conserved cysteine residue [41,70]. Likewise, PEX5-mediated peroxisomal import of pre-thiolase, a PTS2 protein, and its processing in the peroxisomal matrix are also not affected when the ubiquitin-conjugating cascade is blocked, a conclusion that we have recently extended

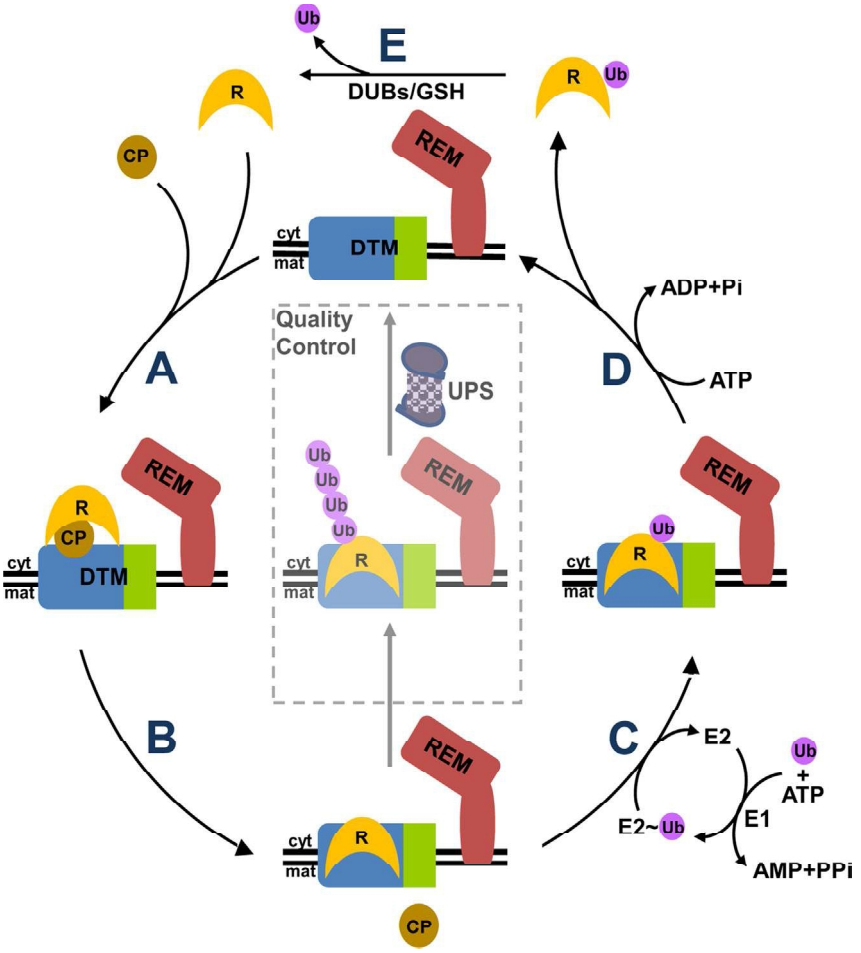

Fig. 1. The roles of ubiquitin in the peroxisome protein import machinery. Peroxisomal matrix cargo proteins (CP) are recognized by cycling receptors (R) in the cytosol. These receptor·cargo protein complexes dock at the peroxisomal membrane docking/translocation module (DTM) (arrow A). The strong protein–protein interactions established between the receptor and DTM components result in the insertion of the receptor into the DTM with the concomitant translocation and release of the cargo protein into the organelle matrix (arrow B). The receptor is then monoubiquitinated at a conserved cysteine residue (arrow C), and extracted back to the cytosol by the ATP-dependent receptor export module (REM) (arrow D). Finally, the ubiquitin moiety is removed probably by a combination of enzymatic (DUBs) and non-enzymatic mechanisms (*e.g.*, by nucleophiles such as glutathione, GSH) (arrow E). When receptors become jammed at the DTM, they are removed and degraded via the ubiquitin-proteasome pathway (UPS).

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also to PTS1 proteins ([35] and unpublished observations). Thus, docking, insertion and cargo release do not depend on mono-ubiquitination of PEX5. These observations strongly suggest that monoubiquitination of PEX5 is required for nothing else other than its export into the cytosol, a step catalyzed by the AAA ATPases of the REM, PEX1 and PEX6.

The mechanistic details of how monoubiquitinated PEX5 and PEX5-related proteins are recognized by the REM are not entirely understood. In principle, the REM could interact directly with DTM-embedded monoubiquitinated receptors. Alternatively, the recognition event might be mediated by an ubiquitin-binding adaptor protein. Some data supporting this last possibility was recently reported [89]. Using an *in vitro* import/export system, the authors noticed that export of peroxisomal PEX5 could be stimulated by adding back cytosolic proteins to the organelle fraction. Purification of this cytosolic activity led to the identification of AWP1, an ubiquitin-binding protein previously shown to interact with a member of the protein kinase C family [90] and to be a regulator of the NF-κB signaling pathway [91,92]. Protein–protein interaction studies led the authors to conclude that AWP1 mediates the interaction of Ub-PEX5 with the REM thus explaining its stimulatory effect on PEX5 export.

Dislocation of DTM-embedded Ub-PEX5 back into the cytosol is followed by its deubiquitination. This step is probably very fast *in vivo* because dithiothreitol-sensitive Ub-PEX5 species can only be detected in organelle fractions [42,49,93]. The DUBs acting on Ub-PEX5 have been recently identified in both yeast (UBP15) and mammals (USP9X) [48,50]. Interestingly, knock-out and knock-down of UBP15 and USP9X genes, respectively, do not lead to an accumulation of Ub-PEX5 in the cytosol, as would be expected if these enzymes were the only mean to remove ubiquitin from PEX5. Clearly, there are alternative ways to deubiquitinate PEX5, which may or may not include other less active/redundant DUBs. Indeed, as proposed recently deubiquitination of PEX5 does not have to necessarily involve a DUB [49]. This is due to the fact that the thioester bond linking ubiquitin to PEX5 is quite labile in the presence of physiologically relevant concentrations of glutathione, displaying a half-life of just 2.3 min. Interestingly, DTM-embedded Ub-PEX5 is resistant to this transthiolation reaction suggesting that such non-enzymatic deubiquitinating mechanism would not create a futile ubiquitination/deubiquitination cycle at the DTM. Deubiquitination of PEX5 completes the PEX5-mediated protein import cycle.

6. Concluding remarks

Our understanding on the PEX5-mediated protein import pathway has increased dramatically in the last decade. The field has clearly moved into the functional/structural characterization of this machinery and we now have at least some ideas, as faint as they may be, on the role played by all components of the PIM. The challenge now is to understand its mechanistic details. Particularly puzzling in the PIM/ubiquitin topic is the fact that monoubiquitination of PEX5 and PEX5-related receptors occurs at a cysteine residue. This would be the expected situation if these receptors were E3-like proteins such as the members of the HECT and RBR E3 family [94]. However, this is clearly not the case: substitution of the conserved cysteine by a lysine (the classical target of ubiquitination) results in a PEX5 protein displaying seemingly normal import/export activities both in *in vitro* and *in vivo* assays [49]. We do know that, in contrast to quality control polyubiquitination, unconventional ubiquitination of receptors occurs at each protein import cycle, meaning that each receptor molecule is probably subjected to hundreds/thousands of monoubiquitination/deubiquitination cycles during its life time. Maybe this property holds the answer to the cysteine enigma. The thiol group of a cysteine residue is a much stronger nucleophile and a better leaving group than the ε-amino group of a lysine. The first property means that ubiquitination at a cysteine residue has the potential to occur at a larger rate than the classical lysine-targeted ubiquitination. This would imply that by using unconventional monoubiquitination of its receptors the PIM could support larger protein import fluxes. We note that previous attempts aiming at detecting a difference between the monoubiquitination rates of PEX5 and a PEX5 protein possessing a cysteine-to-lysine substitution yielded negative results [49]. However, whether or not the PIM was working at its maximum capacity in the assays used in that work remains unknown, and thus a putatively rate-limiting step (*i.e.*, monoubiquitination of the lysine-containing PEX5) might have escaped detection. The fact that the thiol group of a cysteine residue is a good leaving group may also have an impact on the mono-ubiquitination/deubiquitination cycle of the receptors. Indeed, as discussed above, deubiquitination of these thioesters may be achieved simply by a non-enzymatic transacylation reaction of the bound ubiquitin to a physiological relevant nucleophile (*e.g.*, glutathione). As hypothesized before, the existence of redundant deubiquitination mechanisms acting on these receptors might increase their half-lives [49]. Finally, it may be relevant to note that modification of the conserved cysteine of the receptors by any molecule other than ubiquitin would immediately block the DTM. Considering that cysteine residues can be modified in several manners (*e.g.*, oxidized, and acylated), the conserved cysteine could also have a regulatory role functioning, for instance, as a sensor of oxidative stress. In this putative scenario, newly synthesized peroxisomal enzymes (*e.g.*, catalase) would no longer be imported into the organelle and would remain in the cytosol [49,95].

We are still far from understanding how the RING peroxins work, but problems with this family of proteins are clearly not unique to the peroxisome biogenesis field. Naturally, *in vitro* ubiquitination assays using recombinant proteins may provide some of the answers we need, particularly if the substrate-binding subunit(s) of the peroxisomal E3 is(are) included in the assays. The recent findings showing that all three RING peroxins are needed for both monoubiquitination and polyubiquitination of PEX20 [73] should also be considered when performing this type of *in vitro* assays, because they raise the so-far unexpected possibility that the RING peroxins work, not alone, not in pairs, but rather as a trio of modules of a multi-Zn^{2+}-binding domain E3 ligase. Clearly, there is still a long way to go before we understand the molecular details of receptor ubiquitination at the DTM.

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